

DISS. ETH NO. 23732

# **MicroRNAs Contribute to the Protective Actions of 17 $\beta$ -Estradiol on the Vascular System**

A thesis submitted to attain the degree of  
DOCTOR OF SCIENCES of ETH ZURICH  
(Dr. sc. ETH Zurich)

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2016



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# Index

<b>Acknowledgements.....</b>	<b>7</b>
<b>Summary .....</b>	<b>9</b>
<b>Riassunto .....</b>	<b>11</b>
<b>Abbreviations.....</b>	<b>13</b>
<b>1 Introduction .....</b>	<b>17</b>
1.1 Estrogen(s) biology and pharmacology .....	17
1.1.1 Biological effects.....	17
1.1.2 Estrogen synthesis .....	18
1.1.3 Estrogen metabolism .....	19
1.1.4 Estrogen Receptors.....	20
1.1.5 Pathways of estrogen action.....	22
1.2 MicroRNA biology and pathophysiology .....	25
1.2.1 MicroRNAs .....	25
1.2.2 MicroRNA Biogenesis.....	25
1.2.3 Cellular function and mode of action .....	27
1.2.4 Analysis of microRNA function .....	28
1.2.5 Association with diseases.....	29
1.2.6 Association with estrogens .....	30
1.3 Cardiovascular disease .....	31
1.3.1 Atherosclerosis and cardiovascular disease etiology .....	31
1.3.2 The role of vascular cells in vascular remodeling .....	32
1.4 The effects of Estrogens on the cardiovascular system .....	34
1.4.1 Beneficial actions of estradiol on vascular cells.....	34
1.4.2 Molecular mechanisms of estradiol-mediated vascular protection.....	36
1.5 MicroRNAs in cardiovascular biology and disease.....	41
1.5.1 MicroRNAs in endothelial cells .....	42
1.5.2 MicroRNAs in smooth muscle cells .....	42
1.5.3 MicroRNA cell-specific effects .....	43

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<b>2</b>	<b>General Hypothesis and Objectives .....</b>	<b>45</b>
<b>3</b>	<b>Materials.....</b>	<b>49</b>
3.1	Cell culture .....	49
3.2	Chemicals and buffers .....	49
3.3	MicroRNA sequences and primers.....	52
3.4	Antibodies .....	53
3.5	Plastic material.....	54
3.6	Instruments and software .....	54
<b>4</b>	<b>Methods .....</b>	<b>57</b>
4.1	Cell culture .....	57
4.2	MicroRNA sequencing .....	58
4.3	Transfection with microRNA Mimics and Antimirs.....	59
4.4	Cell proliferation studies .....	60
4.5	Migration studies .....	61
4.6	Microvessel formation assay .....	61
4.7	Viability studies .....	62
4.8	Protein expression analysis.....	62
4.9	MicroRNA and pri-microRNA expression analysis .....	63
4.10	Statistical analysis .....	66
<b>5</b>	<b>Results and Discussion.....</b>	<b>67</b>
5.1	E2 regulates miRNAs in ECs .....	67
5.1.1	E2 alters miRNA expression in EPCs .....	67
5.1.2	E2 down-regulates miR-193a in HUVECs .....	68
5.1.3	E2 down-regulates miR-221 expression in HUVECs.....	69
5.1.4	Discussion .....	70
5.2	The role of miR-193a and miR-221 in ECs.....	73
5.2.1	Mimics and Antimirs dysregulate the expression of miRNAs.....	73
5.2.2	miR-193a and miR-221 inhibit HUVEC function .....	76
5.2.3	miR-193a and miR-221 Antimirs mimic the effects of E2.....	79
5.2.4	miR-193a and miR-221 abrogate the protective actions of E2 .....	82
5.2.5	miR-193a and miR-221 do not have additive effects .....	85
5.2.6	Discussion .....	86

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5.3	Intracellular mechanisms of E2-regulated miRNAs in EC .....	91
5.3.1	The role of the PI3K/ Akt pathway in mediating the effects of E2 and miR-193a and miR-221 in vasculogenesis .....	92
5.3.2	The role of VEGF-A in mediating the effects of E2 and miR-193a and miR-221 in vasculogenesis .....	95
5.3.3	The role of ALK1/ SMAD1/5/8/ Id1 pathway in mediating the effects of E2 and miR-193a and miR-221 in vasculogenesis .....	98
5.3.4	Discussion .....	101
5.4	E2 regulates miRNAs in SMCs .....	108
5.4.1	E2 down-regulates miR-193a and miR-221 in HCASMCs.....	108
5.4.2	Discussion .....	109
5.5	The role of miR-193a and miR-221 in SMCs.....	113
5.5.1	Mimics and Antimirs dysregulate the expression of miRNAs.....	114
5.5.2	miR-193a and miR-221 induce HCASMC proliferation and migration ..	116
5.5.3	miR-193a and miR-221 Antimirs mimic the effects of E2.....	119
5.5.4	miR-193a and miR-221 abrogate the protective actions of E2 .....	123
5.5.5	miR-193a and miR-221 do not have additive effects .....	125
5.5.6	Discussion .....	126
5.6	Intracellular mechanisms of E2-regulated miRNAs in SMCs.....	131
5.6.1	miR-221 and miR-193a stimulate G <sub>1</sub> -S phase progression .....	131
5.6.2	miR-221 and miR-193a activate the PI3K/ Akt signaling pathway .....	139
5.6.3	Discussion .....	140
5.7	ER $\alpha$ mediates miR-221 and miR-193a down-regulation .....	146
5.7.1	ER $\alpha$ mediates miRNA down-regulation in HUVECs .....	147
5.7.2	ER $\alpha$ mediates miRNA down-regulation in HCASMCs .....	148
5.7.3	Discussion .....	151
5.8	E2 modulates miR-221 and miR-193a processing .....	153
5.8.1	E2 does not influence pri-miR-221 and pri-miR-193a in HUVECs.....	153
5.8.2	E2 abrogates the effects of PDGF-BB on pri-miRNA expression in HCASMCs .....	155
5.8.3	Discussion .....	157

5.9	Differential effect of miR-193a and miR-221 in vascular ECs and SMCs .....	159
5.9.1	E2 down-regulates miR-193a and miR-221 via ER $\alpha$ .....	159
5.9.2	miR-193a and miR-221 differentially regulate ECs and SMCs .....	160
5.9.3	Down-regulation of miR-193a and miR-221 by E2 mediates its differential actions on vascular cells.....	162
5.9.4	Discussion .....	164
<b>6</b>	<b>Conclusion and Perspectives .....</b>	<b>167</b>
	<b>References .....</b>	<b>171</b>
	<b>Curriculum Vitae .....</b>	Errore. Il segnalibro non è definito.
	<b>Publications and Presentations .....</b>	Errore. Il segnalibro non è definito.

## Acknowledgements

Throughout my PhD I have received support and help from many people. It is with great pleasure that I thank all those who made this thesis possible.

I would like to begin with Prof. Dr. Raghvendra K. Dubey, for giving me the opportunity to do my PhD in his group at the Department of Reproductive Endocrinology. I am grateful for his guidance, the stimulating discussions and the inspiring ideas. His enthusiasm and optimism were always motivating and encouraged me through the tougher times.

To Prof. Sabine Werner my sincere gratitude for supervising my project, for her time invested in my thesis and for providing helpful suggestions and advice.

Many thanks also to Prof. Christian Grimm and Prof. Constance Ciaudo, my co-supervisors, for their valuable scientific feedback at our annual meetings and for their helpful guidance in solving challenging problems.

To all the current and former lab members: Lisa, Yuliya, Federica, Doris and Jasmin. Thank you for your daily help and support in the lab, the scientific discussions and easy-going conversations, daily life advice and sport activities during lunchtimes. I enjoyed working with you in such a nice and friendly atmosphere. Special thanks to Doris and Lisa U., for their patience with me in difficult times and for listening to my excessive complaining in the lab and by phone. Big thanks also to Niki, who helped with the final evaluation of the sequencing data. I am also very thankful to Lisa K., who elucidated the role of ERs, relieving me from lots of RT-qPCRs.

To all the people in Schlieren, especially Matthias, Caroline and Claudio from the Pneumology group for technical help and assistance and Katrin and Theodora for their company at lunch breaks on the rooftop or jogging during the last year.

To the Functional Genomic Center Zürich, especially Lennart Opitz, for running and evaluating the small RNA sequencing experiment.

To my family and friends, who supported and encouraged me through all this time and have made my non-scientific life so wonderful and valuable.

A special thanks to You, who always believed in me. You know who you are.

Finally I would like to thank the Swiss National Science Foundation (31003A-138067) and the Romanian-Swiss Research Program (IZERO 142213/1) and the Hartmann Müller foundation for providing the financial support for the project.



## Summary

The incidence of cardiovascular disease (CVD) is lower in premenopausal women, compared to postmenopausal women and age-matched men. This suggests an association between estrogen levels and vascular protection. This notion is supported by findings of epidemiological studies and by the fact that postmenopausal women taking hormone replacement therapy within 5 years after the onset of menopause have a reduced risk of vaso-occlusive disorders. Indeed, estradiol (E2) inhibits the pathological vascular remodeling processes leading to atherosclerosis and vaso-occlusion. It improves the endothelial integrity and stimulates its recovery/regeneration following damage or dysfunction. Moreover, E2 inhibits the abnormal proliferation and migration of vascular Smooth Muscle Cells (SMCs), thus preventing intimal thickening. The beneficial action of E2 in the vascular system has been intensively studied; nevertheless the molecular mechanisms remain unclear. Recent findings provide evidence that microRNAs (miRNAs) regulate the physiology and pathophysiology of vascular cells and are involved in estrogen signaling. However, their role in mediating the differential effects of E2 in vascular Endothelial Cells (ECs) and vascular SMCs remains undefined.

Hence, in this study we investigated the role of E2-regulated miRNAs in mediating the beneficial actions of E2 in the vasculature. We identified two miRNAs, miR-193a and miR-221, which are down-regulated by E2 in ECs and vascular SMCs. Since endothelial dysfunction and abnormal growth of vascular SMCs contribute to the development of vascular pathologies, we investigated the role of miR-193a and miR-221 in modulating EC and vascular SMC function and the underlying mechanisms. Using synthetic miRNA mimics and miRNA antimirs to respectively up-regulate or inhibit the expression of the identified miRNAs, we demonstrate that they inhibit proliferation, migration and vasculogenesis in Human Umbilical Vein Endothelial Cells (HUVECs). Moreover, we show that these effects are mediated via the inhibition of two key pathways for vasculogenesis, i.e. PI3K/ Akt/ VEGF and ALK1/ SMAD1/5/8/ Id1 signaling. Further, we demonstrate that miR-193a and miR-221 induce proliferation and migration of Human Coronary Artery Smooth Muscle Cells (HCASMCs) and that the increased growth is due to the stimulation of G<sub>1</sub> to S phase progression of the cell cycle. Importantly, we provide evidence that the inhibition of miR-193a and miR-221 by E2 contributes to its beneficial effects in vascular cells and

may explain its differential actions in ECs and vascular SMCs. In addition, we provide evidence that down-regulation of miR-193a and miR-221 requires estrogen receptor (ER)- $\alpha$  and that E2 potentially inhibits the maturation process of these miRNAs, rather than having an effect on the transcription of their precursor molecules.

In summary, our findings demonstrate that miR-193a and miR-221 mediate the differential actions of E2 on vascular ECs and SMCs and that their down-regulation by E2 may prevent vascular remodeling associated with vaso-occlusive disorders in CVD. In conclusion, we postulate that miR-193a and miR-221 may be exciting novel therapeutic targets to treat CVD in postmenopausal women and in men, as miRNA therapeutics may overcome the feminizing and carcinogenic actions of E2.

## Riassunto

La minore frequenza di malattie cardiovascolari in donne giovani rispetto a donne in menopausa o uomini della medesima età, suggerisce un'associazione tra estrogeni e protezione vascolare. Questa teoria è sostenuta da studi epidemiologici e dal fatto che le donne in menopausa, soggette a terapia ormonale sostitutiva entro 5 anni dall'inizio della menopausa, hanno un rischio minore di disturbi vasoocclusivi. Infatti, l'estradiolo (E2) inibisce il rimodellamento vascolare patologico che porta alla formazione di aterosclerosi e la conseguente ostruzione dei vasi sanguigni. L'E2 migliora l'integrità dell'endotelio e stimola la sua rigenerazione in seguito a danni o malfunzionamento. Inoltre l'E2 inibisce la proliferazione e migrazione delle Cellule Muscolari Lisce (CML) vascolari, pertanto impedendo l'ispessimento intinale dei vasi sanguigni. Gli effetti benefici dell'E2 sul sistema vascolare sono stati studiati intensamente; ciò nonostante i meccanismi molecolari restano poco chiari. Recenti scoperte dimostrano che i microRNA (miRNA) regolano la fisiologia e patologia delle cellule vascolari. Inoltre diversi studi attestano che i miRNA sono coinvolti nella trasmissione intracellulare degli estrogeni. Tuttavia rimane incerto il ruolo dei miRNA nella mediazione degli effetti differenziali dell'E2 nelle Cellule Endoteliali (CE) e CML vascolari.

Lo scopo di questa ricerca è di studiare il ruolo dei miRNA regolati dall'E2 nel mediare gli effetti benefici dell'E2 nel sistema vascolare. Abbiamo identificato due miRNA, miR-193a e miR-221, la cui espressione è ridotta dall'E2 sia nelle CE sia nelle CML. Considerando che sia il danneggiamento dell'endotelio che la crescita anormale delle CML contribuiscono allo sviluppo di patologie vascolari, abbiamo esaminato il ruolo svolto da miR-193a e miR-221 in queste cellule. Utilizzando miRNA mimi e antagonisti per aumentare e rispettivamente diminuire l'espressione dei miRNA endogeni, abbiamo dimostrato che miR-193a e miR-221 bloccano la proliferazione, migrazione e formazione di vasi sanguigni nelle CE derivate della vena ombelicale umana (HUVEC). Inoltre abbiamo osservato che questi effetti sono dovuti all'inibizione di due vie di trasmissione intracellulare chiave per la formazione dei vasi sanguigni, più precisamente PI3K/ Akt/ VEGF e ALK1/ SMAD1/5/8/ Id1.

Abbiamo ulteriormente dimostrato che miR-193a e miR-221 inducono la proliferazione e migrazione delle CML derivate da arterie coronarie umane

(HCASMC) e che l'aumento della crescita è dovuto alla stimolazione della transizione dalla fase G<sub>1</sub> a S del ciclo cellulare. La regolazione dei livelli di espressione di miR-193a e miR-221 da parte dell'E2 contribuisce ai suoi effetti benefici nelle cellule vascolari. Questa azione può spiegare la differente regolazione della crescita di CE e CML in seguito a trattamento con E2. Inoltre, proviamo che l'E2 necessita del recettore estrogeno ER $\alpha$  per ridurre l'espressione di miR-193a e miR-221. Infine proviamo che l'E2, in concomitanza con ER $\alpha$ , blocca il processo di maturazione di entrambi i miRNA, piuttosto che ostacolarne la trascrizione dal DNA.

Riassumendo, i nostri risultati dimostrano che miR-193a e miR-221 mediano l'azione differenziale dell'E2 nelle CE e CML vascolari e indicano che la riduzione dei loro livelli di espressione dovuta all'E2 impedisce il rimodellamento vascolare comunemente associato alle affezioni ostruttive delle malattie cardiovascolari. In conclusione ipotizziamo che miR-193a e miR-221 potrebbero rappresentare ottimi bersagli per nuove terapie contro le malattie cardiovascolari, sia nelle donne in menopausa sia negli uomini. Infatti, una terapia basata sui miRNA potrebbe revocare gli effetti indesiderati causati dall'E2, come la femminilizzazione dei soggetti maschili e l'aumento del rischio di cancro nei soggetti femminili.

## Abbreviations

2ME	2-Methoxyestradiol
2OHE	2-Hydroxyestradiol
A	Antimir
A193a	miR-193a Antimir
A221	miR-221 Antimir
AC	Control Antimir
AGO	Argonaute
Akt	Protein kinase B
ALK1	Activin Receptor-like Kinase 1
ALK1Fc	ALK1 neutralizing antibody
BrdU	5'-Bromo-2'-deoxyuridine
Ca <sup>2+</sup>	Calcium
cAMP	3',5'-cyclic Adenosine Monophosphate
CDK	Cyclin-Dependent Kinase
cGMP	3',5'-cyclic Guanosine Monophosphate
COMT	Catechol-O-methyltransferase
CVD	Cardiovascular Disease
CYP450	Cytochrome P450 enzyme
DGCR8	DiGeorge Syndrome Critical Region 8
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DPN	Diaryl-Proprio-Nitrile, ER $\beta$ specific agonist
E1	Estrone
E2	17- $\beta$ Estradiol
E3	Estriol
EC	Endothelial Cell
ELISA	Enzyme-Linked Immunoassay
eNOS	endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cell
ER	Estrogen Receptor
ERE	Estrogen Response Element
ERK1/ 2	Extracellular Signal-Regulated Kinases 1/ 2

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EtOH	Ethanol
FGF	Fibroblast Growth Factor
G <sub>0</sub>	Cell cycle phase Gap 0
G <sub>1</sub>	Cell cycle phase Gap 1
G1	GPER specific agonist
G <sub>2</sub>	Cell cycle phase Gap 2
GPCR	G-protein Coupled Receptor
GPER	G-protein Coupled Estrogen Receptor
GTP	Guanosine Tri-Phosphate
HCASMC	Human Coronary Artery Smooth Muscle Cell
HERS	Heart and Estrogen/ Progestin Replacement Study
hr	hours
HRT	Hormone Replacement Therapy
HSD	Hydroxysteroid Dehydrogenase
HUVEC	Human Umbilical Vein Endothelial Cell
ICI	ICI 182-780, fulvestrant, ER unspecific antagonist
Id1	Inhibitor of Differentiation 1
LDL	Low-density Lipoprotein
LY	LY294002, PI3K-inhibitor
M	Mimic
M193a	miR-193a Mimic
M221	miR-221 Mimic
MC	Control Mimic
MCF-7	Hormone-responsive breast cancer cell line
Mg <sup>2+</sup>	Magnesium
miRNA	microRNA
MPP	Methyl-Piperidino-Pyrazole, ER $\alpha$ unspecific antagonist
mRNA	messenger Ribonucleic Acid
MTT	Thiazol Blue Tetrazolium Bromide
NO	Nitric Oxide
p27	p27 (Kip1), cyclin dependent kinase inhibitor
PDGF	Platelet-derived Growth Factor
PI	Propidium Iodide
PI3K	Phosphoinositide 3 Kinase

PPT	Propy Pyrazole Triol, ER $\alpha$ specific agonist
pRB	hyperphosphorylated Retinoblastoma protein
Pre-miRNA	precursor microRNA
Pri-miRNA	primary microRNA
RB	Retinoblastoma protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rpm	rounds per minute
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
S	Cell cycle phase Synthesis
Ser	Serine
Skp2	S-phase kinase associated protein 2
SMC	Smooth Muscle Cell
TGF $\beta$	Transforming Growth Factor Beta
Thr	Threonine
Tyr	Tyrosine
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VEGFAb	VEGF neutralizing antibody
WHI	Women`s Health Initiative





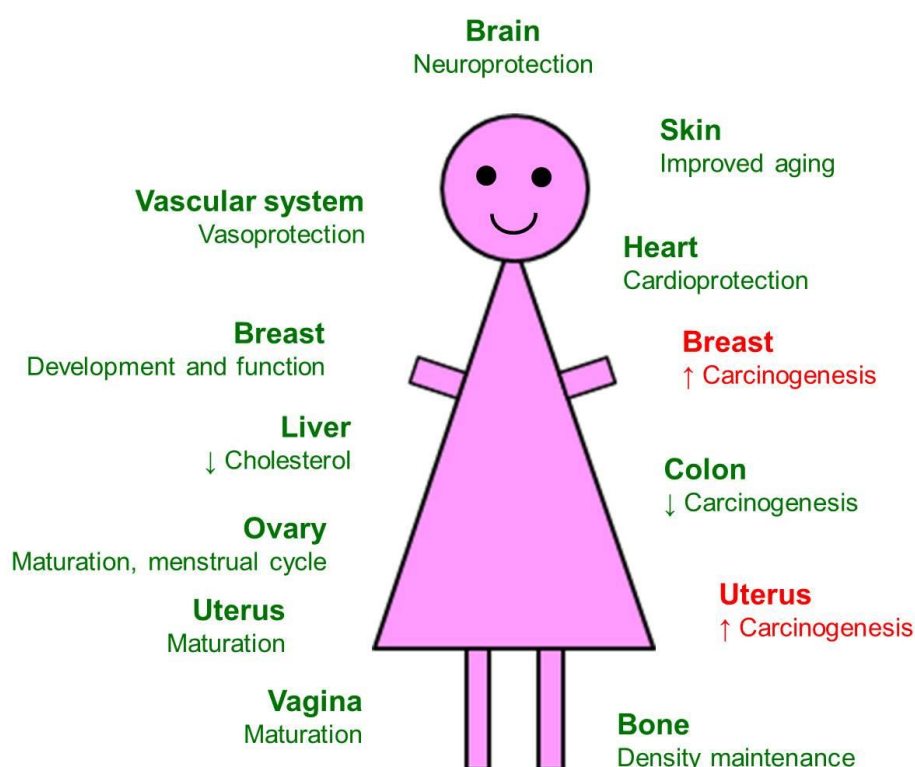
# 1 Introduction

## 1.1 Estrogen(s) biology and pharmacology

Estrogen is the generic term for the primary female sex hormones, which include the endogenous forms Estrone (E1), 17- $\beta$ -Estradiol (E2) and Estriol (E3). Among these, E2 is functionally the most relevant as it is predominant in women of reproductive age. E1 is the weaker form of estrogen and the only one produced after menopause. E3 is only produced in significant quantities during pregnancy [1].

### 1.1.1 Biological effects

Estrogens are important for the growth and function of the female reproductive system. They promote the development of secondary sex characteristic, regulate the menstrual cycle and influence the fertilization process and maintenance of pregnancy. In addition to these classical functions, estrogens play a critical role in regulating the physiology of many other tissues (**Figure 1**).



**Figure 1:** A simple model of the positive (green) and negative (red) effects of estrogens in the female body.

Among the potential positive effects, estrogens have neuroprotective effects in the brain and may prevent Alzheimer`s disease, stroke and Parkinson`s disease [2, 3]. In the vascular system they improve vascular function and protect against cardiovascular diseases (CVDs). Additionally, estrogens ameliorate cholesterol metabolism by the liver, increase bone density thereby protecting against osteoporosis, improve skin aging, and reduce the risk of colon cancer [4, 5]. On the other hand the deleterious effects of estrogens include increased carcinogenesis in the uterus and in the breast tissue [6]. Interestingly, even though estrogens are considered to be the female hormones, they influence the reproductive system and the fertility in male too [7].

### **1.1.2 Estrogen synthesis**

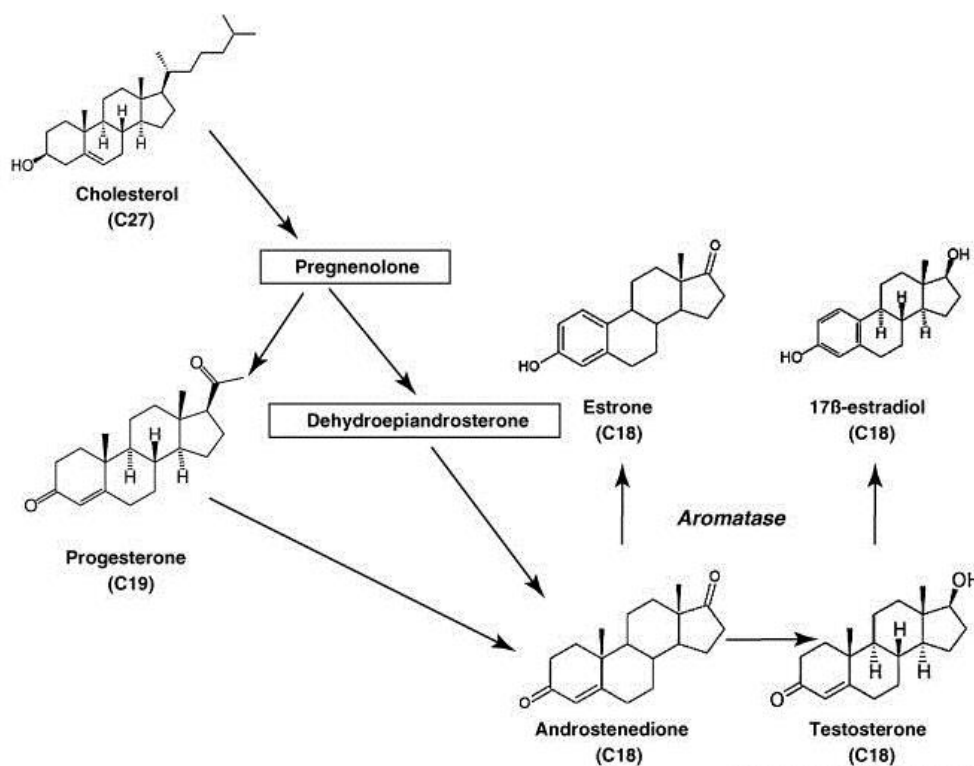
The ovaries are the main location for estrogen production [8], but other tissues are also capable of estrogen synthesis, for example brain, liver, adipose tissue, skin, muscle, breast, vaginal mucosa and endometrium.

Estrogens, as well as the other endogenous steroid hormones are synthesized from cholesterol [9] as described in **Figure 2**. First, cholesterol is taken up into steroidogenic cells, in which it is converted to pregnenolone by specific Cytochrome P450 (CYP450s) enzymes. Then pregnenolone is enzymatically processed to testosterone via two different mechanisms: either through production of progesterone by the enzyme 3- $\beta$ -Hydroxysteroid Dehydrogenase (HSD) and conversion of progesterone to androstenedione, or through hydroxylation at the 17- $\alpha$  position by the enzyme 17- $\alpha$ -hydroxylase to form 17- $\alpha$ -pregnenolone, which is further converted to dehydroepiandrosterone by the enzyme 17,20 lyase and finally converted into testosterone by the 17- $\beta$ -HSD. Finally, testosterone is converted to 17- $\beta$ -estradiol (E2) by the enzyme aromatase.

The ovarian granulosa cells express the highest levels of aromatase in premenopausal women, but it is also expressed by a large number of other tissues, for example adipose tissue, skin, vaginal mucosa, endometrium, breast, liver and vascular cells [10].

Following synthesis, E2 is released into the circulation, where 2-3% of the total amount can be found in a free form and the majority is bound to albumin or sex-hormone binding globulin. Circulating E2 concentrations range from 0.36 nM in the

follicular phase to 2.8 nM during ovulation. The highest levels of circulating E2 are during pregnancy, reaching up to 70 nM, whereas they drop to 0.02 nM following menopause and are comparable to estrogen concentrations in men [11].



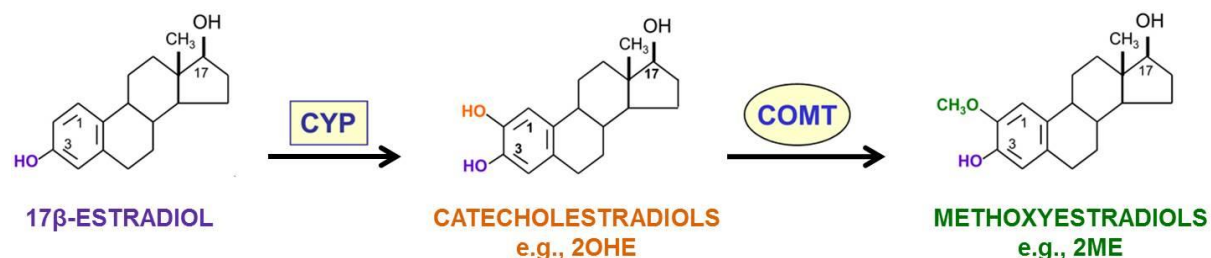
**Figure 2:** Estrogen synthesis from cholesterol. Depicted from Cignarella et al. [9].

### 1.1.3 Estrogen metabolism

Estrogen elimination is facilitated through its conversion to water-soluble metabolites, which can be excreted via urine or feces, via conjugation to sulfates, glucuronides or fatty acids esters. In addition, E2 can be metabolized into oxidative biologically active products as described in **Figure 3**. CYP450 oxidizes E2 to Catecholestrodiols (for example 2-Hydroxyestradiol), which are further metabolized to Methoxyestradiols by Catechol-O-Methyl-Transferase (COMT) [12].

E2 is largely metabolized in the liver, although the enzymes responsible for E2 metabolism (CYP450 and COMT) also occur in other tissues, like vasculature, kidneys, gastrointestinal tract, spleen, brain and pancreas. Though the majority of the E2 metabolites are excreted, there is increasing evidence that they have significant growth regulatory properties in several tissues. In fact, 4-Hydroxyestradiol has carcinogenic effects, whereas 2-Hydroxyestradiol (2OHE) and 2-Methoxyestradiol (2ME) have been shown to inhibit tumor growth [13]. Further, 2ME has been reported

to play a role in vascular cells, since it reduces neovascularization and prevents neointima formation by inhibition of vascular Smooth Muscle Cell (SMC) proliferation [14, 15].



**Figure 3:** 17- $\beta$ -estradiol conversion into the biologically active metabolites 2OHE and 2ME. CYP, Cytochrome P450; 2OHE, 2-Hydroxyestradiol; COMT, Catechol-O-Methyl-Transferase; 2ME, 2-Methoxyestradiol. Depicted and modified from Dubey et al. [16].

### 1.1.4 Estrogen Receptors

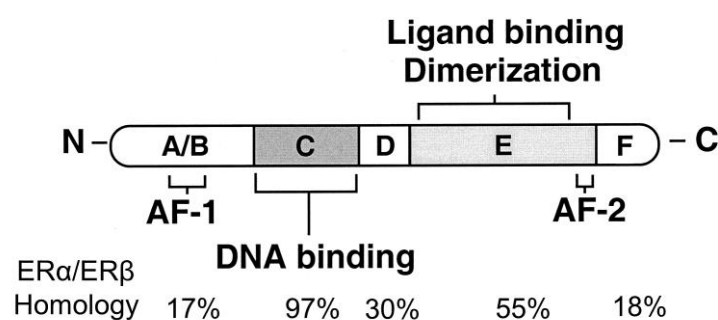
The biological effects of estrogens are largely mediated via Estrogen Receptors (ERs). To date, three ERs have been identified: the classical nuclear receptors ER $\alpha$  and ER $\beta$  and the membrane bound G-protein Coupled ER (GPER).

#### 1.1.4.1 Estrogen Receptor $\alpha$ and Estrogen Receptor $\beta$

ER $\alpha$  was discovered in 1960 and cloned for the first time in 1986 [17], while ER $\beta$  was detected later in 1996 [18]. The receptors are encoded by genes, which are located on different chromosomes (6 and 14, respectively) and encode for proteins containing 595 and 530 amino acids, respectively [19].

ER $\alpha$  and ER $\beta$  belong to the superfamily of nuclear receptors, with 95% located in the cell nucleus and the remaining 5% in the cytoplasm [20]. They are composed of 6 conserved domains A-F, as shown in **Figure 4**. The N-terminal A/B and the C-terminal F domain contain the transactivation function (AF-1 and AF-2 respectively), which interact with co-repressors and co-activators to regulate transcription. The highly conserved C domain is the DNA-binding domain. The D domain is a hinge region that connects the C and E domains. Finally, the E region contains the ligand-binding domain, which confers ligand specificity to the receptor [21]. Although the DNA-binding domains of the two receptor subtypes are very similar (97%), the overall homology is low. Nevertheless, the binding affinity to the major ligand E2 is very similar ( $K_d=0.6$  nM) [18].

Beside the differences in structure and ligand-binding specificity, the tissue distribution of the two classical ERs is also different. For example, ER $\alpha$  expression prevails in the endometrium, breast cancer cells and ovary, whereas ER $\beta$  is predominantly expressed in the kidney, intestinal mucosa, lungs, bone and prostate [4]. Moreover, the ERs show a specific cell type dependent distribution and knock-out studies for ER $\alpha$  and ER $\beta$  provide evidence for differential functions for the two receptors [22]. These findings indicate that the final effect of ER-ligands depends on ER regulation within a cell type, which is influenced by extracellular signals. In this regard, E2 has been shown to regulate the expression of the ERs in many cell types, such as human breast cancer cells [23], retinal pigment epithelial cells [24] and osteoclasts [25].



**Figure 4:** Structure of estrogen receptor. Adapted from Aranda and Pascual [26].

#### 1.1.4.2 G-protein Coupled Estrogen Receptor

The G-protein Coupled ER (GPER) was first discovered between 1996 and 1998 and named GPR30, due to its similarities to the classical seven-transmembrane G-Protein Coupled Receptors (GPRs). Its relation to estrogens was unknown, until in 2000 Filardo et al. discovered that GPR30 is necessary for estrogen-mediated stimulation of ERK1/2 [27]. Only in 2005, the affinity of estrogen to GPR30 was demonstrated by Revankar et al. [28] and Thomas et al. [29] and E2 was identified as the endogenous ligand for this receptor, which nomenclature was finally changed in 2007 to GPER [30].

The *GPER* gene is located on chromosome 7 and its protein entails 375 amino acids [31]. Although the exact structure of GPER is still unknown, it is divided into three parts similar to the GPRs: the extracellular region (N-terminus and three extracellular loops ECL1-ECL3), the transmembrane region consisting of seven  $\alpha$  helices (TM1-TM7) and the intracellular region (three intracellular loops ICL1-ICL3, an amphipathic

helix H8 and the C-terminus). The cellular location of GPER is under debate: it was found to be a membrane receptor in some studies [29, 32], while others suggest its intracellular location within the Endoplasmatic Reticulum [28, 33].

Recent studies have demonstrated the expression and functions of GPER in the reproductive, endocrine, urinary, nervous, immune, musculoskeletal and cardiovascular system [34].

### **1.1.5 Pathways of estrogen action**

Estrogen actions can be exerted via genomic pathways, resulting in changes in gene expression either through direct activation of the ERs as transcription factors (classical pathway), via association with other transcription factors (indirect, ERE-independent pathway) or without the involvement of estrogens (ligand-independent pathway). Additionally, there is evidence that E2 induces its biological effects also via non-genomic mechanisms [35].

#### **1.1.5.1 Genomic pathways**

##### *Classical (direct) pathway*

In the classical pathways of E2 action, the lipophilic E2 diffuses into the cells through the membrane and binds to the intracellular ERs. Upon E2 binding to the receptor, a conformational change takes place and the ER dissociates from the heat shock proteins (chaperones), which stabilize the receptor and mask the DNA-binding site in the absence of ligand binding. Homo- or heterodimerization of ER $\alpha$  and ER $\beta$  allows the E2-ER complex to diffuse into the nucleus, where it binds to the DNA at specific Estrogen Response Elements (EREs), located within the regulatory regions of target genes. Finally, inhibition or induction of the target genes is regulated by the association of the E2-ER complex with several co-repressors and co-activators via the AF-1 and AF-2 domains [36] (**Figure 5A**).

##### *ERE-independent pathways*

Several studies provide evidence that E2-ER complexes are able to control expression of genes lacking the ERE sequence in their regulatory regions. In this case, after ligand activation ERs interact with other transcription factors, such as Fos, Jun and Sp1, thereby affecting target regulation by indirect DNA binding [37]. Some

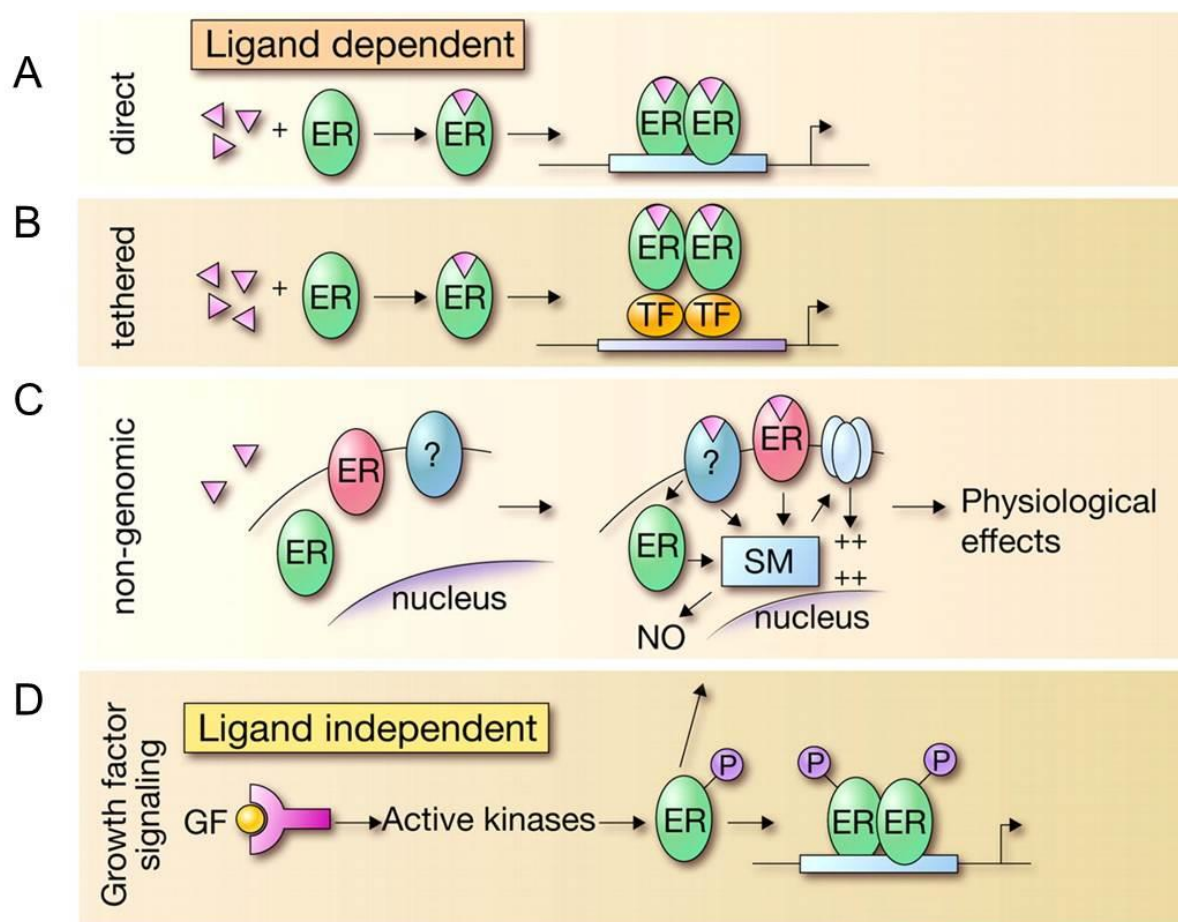
examples are the genes encoding *Epidermal Growth Factor (EGF)*, *EGF receptor* and *cyclin D1* [38] (**Figure 5B**).

#### *Ligand-independent pathways*

Activation of ERs is also possible through other signaling pathways without the binding of a ligand. In this case, growth factor activated kinases phosphorylate ERs, stimulating them to dimerize and directly regulate gene expression [39] (**Figure 5D**).

#### **1.1.5.2 Non-genomic pathway**

The non-genomic pathway is not as well understood as the genomic mechanisms, even though it has been detected in many tissues. For example, the effects of estrogens are observed within seconds to minutes following stimulation and are not explainable by mechanisms involving genomic changes, which require hours to days. The ligand binds and activates a receptor at the membrane, potentially involving GPER [29, 32], which initiates signaling cascades, including involvement of second messengers (**Figure 5C**). Examples of rapid physiological response of E2 include the modulation of the activity of Mitogen-Activated Protein Kinases (MAPKs) ERK1 and ERK2 [40], increase in Nitric Oxide (NO) synthesis via stimulation of endothelial Nitric Oxide Synthase (eNOS) [41], activation of cAMP formation by Protein Kinase A (PKA) and Protein Kinase C (PKC) activation and increase in cellular  $\text{Ca}^{2+}$  [42].



**Figure 5:** Model representing the mechanistically distinct molecular pathways used in the regulatory actions of ERs. **Panel A:** The classic (direct) pathway includes ligand activation and a direct DNA binding to ERE before modulation of gene regulation. **Panel B:** The tethered ERE-independent pathway includes protein-protein interaction with other TFs after ligand activation, and thereby gene regulation is affected by indirect DNA binding. **Panel C:** Non-genomic pathway. The ligand activates a receptor and signaling cascades are initiated via SMs ultimately leading to a rapid physiological response without involving gene regulation. **Panel D:** The ligand-independent pathway includes activation through other signaling pathways, like growth factor signaling. In this case, activated kinases phosphorylate ERs and thereby activate them to dimerize, bind DNA, and regulate genes. *ER*, Estrogen Receptor; *ERE*, Estrogen Response Element; *TF*, Transcription Factor; *SM*, Second Messenger; *NO*, Nitric Oxide. Depicted from Heldring et al. [43].



## 1.2 MicroRNA biology and pathophysiology

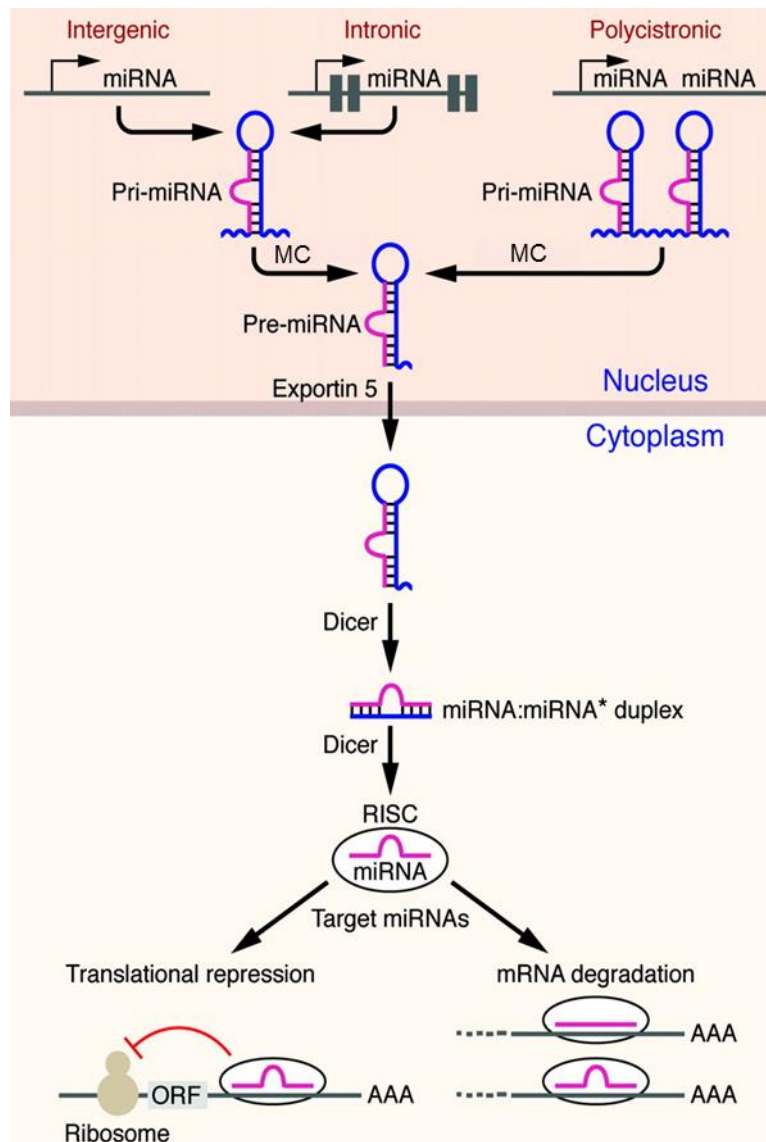
### 1.2.1 MicroRNAs

RNA molecules perform many critical tasks in the cell apart from the classical function as intermediate carriers of genetic information. MicroRNAs (miRNAs), small untranslated RNA molecules containing around 22 nucleotides, are part of these non-coding RNAs and play a widespread role in regulating gene expression at the post-transcriptional level. To the present day, over 1000 miRNAs have been found to be encoded in the human genome [44] and they appear to target at least 60% of all human and mammalian protein-coding genes, thus being involved in many clinically relevant biological and pathophysiological processes [45, 46].

The first miRNA (cel-lin-4) was discovered independently by two research groups studying the development of *C.elegans* in 1993 [47, 48] and followed by a second miRNA (cel-let-7) in 2000 [49]. Only in 2001, small RNAs were recognized as a distinct class of biological regulators and nominated miRNAs [50]. Since then, the number of studies addressing the biology, function and pathophysiology of miRNAs has increased exponentially.

### 1.2.2 MicroRNA Biogenesis

miRNAs are transcribed by RNA polymerase II (RNAPII) either from individual miRNA genes (intergenic), from introns of protein-coding genes (intronic) or from units encoding multiple miRNAs (polycistronic) [51]. Most of the miRNAs are intergenic and have their own promoter, thus likely to be transcribed as independent units [52], while the intronic miRNAs are usually transcribed together with their host genes [53]. As shown in **Figure 6**, following synthesis, the resulting primary miRNA precursor (pri-miRNA) is capped, polyadenylated and processed, first by the Microprocessor Complex (MC) in the nucleus and subsequently by the endonuclease Dicer, following its export to the cytoplasm [54, 55]. The MC is minimally composed of one Drosha protein and two DGCR8 (DiGeorge Syndrome Critical Region 8) proteins [56], which recognize the pri-miRNAs and direct the RNase domain of Drosha to cleave them into 70 to 100 nucleotides long hairpin-shaped precursors (pre-miRNA) [57]. In addition to the minimal MC components, other cofactors may also associate with the complex to mediate the activity of Drosha [58, 59].



**Figure 6:** A schematic representation of miRNA biogenesis and their mode of action. *Pri-miRNA*, primary miRNA; *pre-miRNA*, precursor miRNA; *MC*, Microprocessor Complex; *RISC*, RNA-Induced Silencing Complex; *ORF*, Open Reading Frame. Adapted from van Rooij [60].

Some pre-miRNAs, called Mirtrons, are spliced directly out of very short intronic regions, thus bypassing Drosha-mediated processing [61]. Pre-miRNAs are exported from the nucleus to the cytoplasm by exportin-5 in complex with Ran-GTP [62]. In the cytoplasm, miRNA maturation is continued by the RNase Dicer. Dicer cleaves the pre-miRNA hairpin to form a miRNA:miRNA\* duplex of about 20 to 22 nucleotides in length. Even though both strands of the duplex potentially act as a functional miRNA, usually only one, the functional guide strand, is incorporated into the RNA-Induced Silencing Complex (RISC), while the other, the passenger strand, is subsequently degraded [63]. The RISC finds the target mRNAs by searching for complementary

nucleotide sequences, which is considerably eased by the Argonaute 2 protein (AGO2), a component of RISC, which positions the miRNA optimally for base-pairing to other RNA molecule [64].

### **1.2.3 Cellular function and mode of action**

The main function of miRNA is gene regulation, through base-pairing to mRNAs. Upon binding of miRNA and mRNA, silencing occurs either by translational repression or induction of mRNA degradation, depending on the level of sequence complementarity between the miRNA and the target mRNA. Complete base-pairing, which is unusual in humans but common in many plants, leads to direct cleavage of the mRNA by the AGO2 protein ("slicing"), which results in degradation of the mRNA and release of the miRNA-RISC complex. In case of partial complementarity, silencing is slicer-independent and achieved through translational repression, which is likely to be caused by the disruption of translation initiation [65]. Moreover, recent evidence indicates that P-bodies are essential for miRNA-mediated gene silencing and may contribute to the complexity of its mechanisms, including the reversibility of mRNA silencing [66].

The extent of base-pairing for partial complementarity is typically at least 7 nucleotides, it is called seed sequence in the miRNA and most often occurs in the 3' untranslated region (UTR) of the target mRNA. Interestingly, a single miRNA can regulate a whole set of different mRNAs, given that they have at least partial complementarity to the miRNA seed sequence in their UTRs. Furthermore, regulation by miRNA can be combinatorial as different miRNAs can bind to the same target mRNA to further reduce its translation [67].

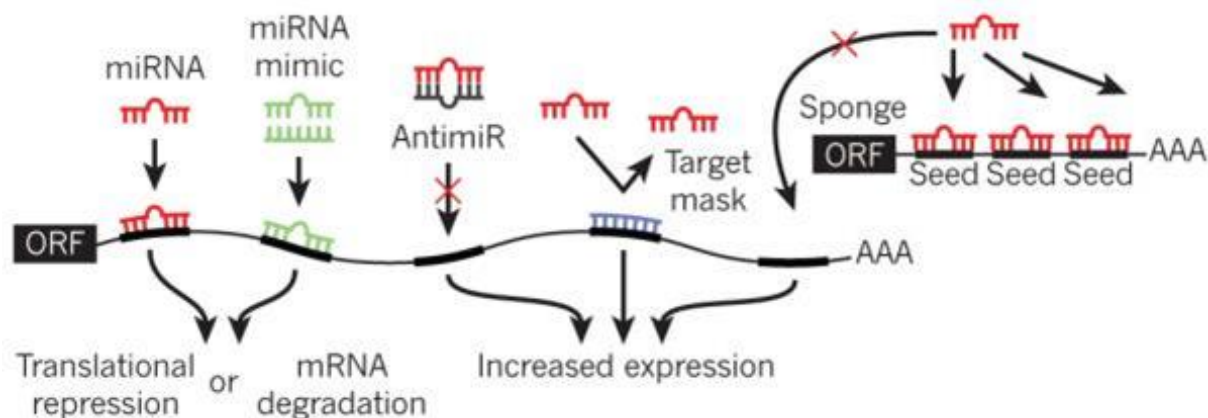
#### **1.2.3.1 Activation of translation**

Down-regulation of the target mRNA is the common mechanism of miRNA-mediated gene regulation; however, there is increasing evidence indicating that miRNA action includes both repression and stimulation, as a result to particular cellular conditions, cell type and context and that both up- and down-regulation can vary from fine tuning to extensive deregulation of gene expression [68, 69]. For instance, miR-145 inhibits ROCK1 expression in osteosarcoma [70], yet mediating myocardin up-regulation during muscle differentiation [71]. Further, KLF-4 is up-regulated by miR-206 in

confluent and non-tumor cells, whereas it is down-regulated by miR-344 in proliferating and normal cells [72].

#### 1.2.4 Analysis of microRNA function

Through the regulation of gene expression, miRNAs are able to modulate most, if not all, of the biological processes which take place in the cells and tissues. Accordingly, the most common strategy to probe miRNA functions are by gain- and loss-of-function approaches as shown in **Figure 7**.



**Figure 7:** The various methods to artificially modulate miRNA expression or activity. Endogenous miRNAs are depicted in red, miRNA Mimic is green, Antimir is shown in grey, the target mask is blue and the miRNA sponge is the transcript with multiple miRNA seed sequences. Depicted from Small and Olson [73].

The expression and activity of a specific miRNA is artificially increased by the delivery of a miRNA Mimic, a chemically modified and stabilized ribonucleotide duplex, which imitates the action of the endogenous miRNA and further down-regulates target gene expression. In contrast, miRNA loss-of-function can be achieved via three different methods. The widely used antisense oligonucleotides complementary to an endogenous miRNA (Antimirs) prevent the miRNA from binding to the seed sequence of the target transcripts, de-repressing the expression of all target mRNAs. In a second approach overexpression of an exogenous mRNA containing multiple copies of the specific miRNA seed sequence (miRNA sponge) competes for miRNA binding and prevents it from base-pairing with the target mRNAs. The third strategy rescues one particular mRNA from miRNA-mediated repression through the delivery of an antisense oligonucleotide able to associate with the miRNA target sequence (target mask) [73].

### 1.2.5 Association with diseases

MiRNAs play an important role in regulating gene expression in normally functioning cells, hence influencing numerous cellular and developmental processes in many cell types, as for example cell differentiation, proliferation, migration and apoptosis. Accordingly, deregulation or abnormal function of miRNAs is linked to abnormal function of the biological processes they regulate and is therefore associated with pathologies [74]. The first human disease associated with deregulated miRNAs was chronic lymphocytic leukemia in 2002 [75], which was followed by many other cancer types, inflammatory diseases, CVDs, kidney diseases, nervous system and metabolic disorders [76], suggesting that miRNAs could serve as therapeutic targets [77]. Indeed, the dysregulated miRNAs can be potentially restored to their original expression levels by miRNA replacement or inhibition strategies.

Company	Targeted miRNA	Diseases	Technology/chemistry	Mechanism/effect	Stage
Regulus Therapeutics	miR-122	HCV infection	Anti-miR	Block HCV infection	Preclinical
	miR-10b	Glioblastoma	Anti-miR	Reduces proliferation by blocking cell cycle progression and triggering cell death	Preclinical
	miR-221	HCC	Anti-miR	Delayed tumor progression resulting in a survival rate	Preclinical
	miR-21	Renal fibrosis	Anti-miR	Reducing the expression of extracellular matrix proteins	Preclinical
	miR-33	Atherosclerosis	Anti-miR	Regulation of cholesterol and fatty acid homeostasis via decrease in very LDL triglycerides and an increase in high-density lipoprotein	Completed preclinical
Santaris Pharma Mirna Therapeutics	miR-122	HCV	Anti-miR	Prolonged mean reductions in viral plasma RNA levels from baseline	Phase IIa
	miR-34	Primary liver cancer or solid cancers with liver involvement	mimic	Reduction in the expression of oncogenes, tumor regression, enhanced the survival, and inhibited the growth of other nonhepatic tumors	Phase I
	miR-155	Hematological malignancies	Anti-miR	Restores normal function and reduces the aberrant cell proliferation	Completed preclinical
miRagen Therapeutics	miR-92	Peripheral artery disease	Anti-miR	Enhances blood vessel growth and improves functional recovery of damaged tissue	Preclinical
	miR-15	Myocardial infarction	Anti-miR	Reduces heart muscle cell death and promotes heart muscle cell regeneration	Preclinical

miRNA=MicroRNAs, HCV=Hepatitis C virus, HCC=Hepatocellular carcinoma, LDL=Low-density lipoprotein

**Figure 8:** Overview of miRNAs therapeutics development status. Depicted from Christopher et al. [78].

Consequently, a small number of miRNAs have entered the preclinical and clinical stage (**Figure 8**) and might be soon available in the market [78]. Furthermore, thanks to their differential expression pattern in diseases and their presence and stability in extracellular fluids such as human plasma and serum, aberrantly expressed miRNAs are also potential diagnostic and prognostic markers [79]. Some examples are the cardiac-specific miR-208a, which is elevated in plasma of acute myocardial infarction patients within 4 h of the onset of the symptoms [80], the 5 specific miRNAs (miR-21, -92, -93, -126 and -29a) overexpressed in the serum from ovarian cancer patients

[81] and the miR-34a, which has been identified as a prognostic marker in non-small-cell lung cancer patients and correlated with relapse and overall survival [82].

### 1.2.6 Association with estrogens

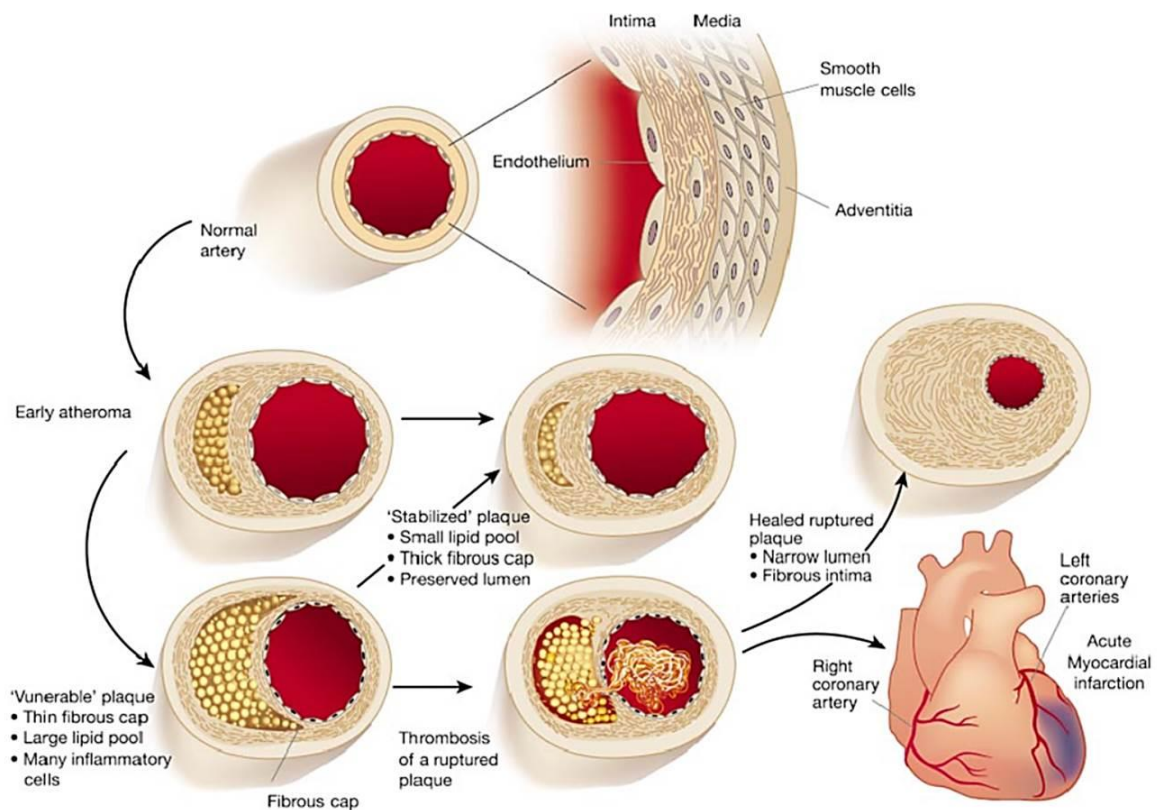
Association between miRNA expression and estrogens was first reported in 2005 [83]. Subsequently the first evidence demonstrating miRNA regulation by E2 was published two years later demonstrating that E2 suppresses miR-206 in MCF-7 human breast cancer cells [84]. To date, over 200 E2-regulated miRNAs have been identified, mostly using microarray [85-88]. Several reports provide evidence that E2 suppresses or stimulates miRNA expression in different human cell lines and tissues [89]. Moreover, changes in expression of E2-regulated miRNAs are linked with estrogen-dependent cellular responses. For instance, in MCF-7 cells E2 significantly induced the expression of the cell proliferation and survival genes *bcl-2*, *cyclin D1* and *survivin* by suppressing the levels of miR-16, miR-143, miR-203 [90]. Furthermore, a broad set of miRNAs, including miR-21, miR-26a, miR-181a, miR-193a, miR-203, miR-23a, miR-221 and miR-222, decreased in MCF-7 cells treated with E2 [91, 92]. Moreover, enforced overexpression of several of the repressed miRNAs reduces E2-dependent cell growth [93]. Since E2-regulated miRNAs actively participate in E2 mediated responses, miRNAs are postulated to play a critical role mediating the regulation of gene expression by E2 and mediating its biological actions [88]. Although estrogenic miRNA regulation has been mainly studied in traditional estrogen target tissues, such as breast and breast cancer cells, endometrial cells and uterus, evidence of E2-regulated miRNAs in other systems, including the vascular system, are emerging. Indeed, E2 induces miR-203, which mediates the inhibitory effects of E2 on vascular SMC proliferation [94].

## 1.3 Cardiovascular disease

### 1.3.1 Atherosclerosis and cardiovascular disease etiology

The leading cause of death and disability in the Western civilization is cardiovascular disease (CVD) [95]. CVD includes numerous pathologies, for instance coronary artery disease, myocardial infarction, heart disease, thrombosis and stroke; however, most of the vascular problems are related to atherosclerosis. The processes leading to atherosclerosis are triggered by the disruption of the functionally active endothelium [96]. Injured Endothelial Cells (ECs) secrete cytokines and express adhesion molecules, thus triggering the inflammatory responses and recruiting monocytes to the vessel wall. These monocytes migrate into the subendothelial space, where they differentiate into macrophages, which ingest low density lipoproteins (LDL) resulting in the formation of foam cells. In addition, damage or dysfunction of the ECs activates secretion of growth factors by platelets, such as Platelet Derived Growth Factor (PDGF)-BB and Transforming Growth Factor (TGF)- $\beta$ . These mitogens, together with autocrine/paracrine factors produced by foam cell and ECs, stimulate vascular SMC proliferation, cell migration into the damaged area and production of extracellular matrix rich in collagen [97]. Moreover, plaque development is enhanced by macrophage proliferation and cytokine production, which further increases the inflammatory response and thickening of the vessel wall. Finally, the evolution of atherosclerosis depends on many factors, though it can be summarized by two scenarios as described in **Figure 9**: the vessel occlusion can be steady, in case of stabilized or healed ruptured plaques, or plaque rupture leads to thrombotic complications, including ischemic stroke, myocardial infarction and death [98].





**Figure 9:** Schematic representation of the evolution of atherosclerosis. Depicted from Libby [98].

### 1.3.2 The role of vascular cells in vascular remodeling

Atherosclerosis develops from a set of many different factors, yet the main participants are ECs and vascular SMCs, as they are directly involved in vascular remodeling processes.

#### 1.3.2.1 Endothelial cells

As described in the previous chapter, atherosclerosis originates from damage or dysfunction of the endothelium, thus highlighting that EC function is imperative for vascular health. Therefore, accelerated recovery or substitution of injured cells might prevent the progression of atherosclerosis [96]. The processes that characterize EC function are viability, proliferation, migration and the ability to form new vessels via vasculogenesis and/ or angiogenesis, in order to supply the tissues with the necessary oxygen and nutrients [99]. Angiogenesis is the formation of new blood vessels from pre-existing vessels, while vasculogenesis designates the de novo



production of vessels [100-102]. Angiogenesis occurs both during embryonic development and in adults, whereas post-natal vasculogenesis is recognized only since 1997, once circulating Endothelial Progenitor Cells (EPCs) were first isolated and described [103]. Importantly, both angiogenesis and vasculogenesis contribute to cardiovascular regeneration, as both processes promote wound healing and neovascularization of injured tissues. Accordingly, the widespread model to assess EC function is through vasculogenesis assays using Human Umbilical Vein ECs (HUVECs) [104].

#### **1.3.2.2 Smooth muscle cells**

The second cell type involved in vascular remodeling are vascular SMCs, which promote the development of atherosclerosis through abnormal proliferation, migration and differentiation, stimulating plaque formation and neointimal thickening [105]. Moreover, vascular SMC growth plays a key role in the development of restenosis following balloon angioplasty [106]. Vascular SMC proliferation and migration are mainly triggered by cytokines and growth factors released from injured ECs. This highlights the importance of a healthy and functional endothelium to maintain vascular SMCs quiescent [99]. Lastly, a controlled and balanced cross-talk between ECs and vascular SMCs is critical for the preservation of functional blood vessels [107].

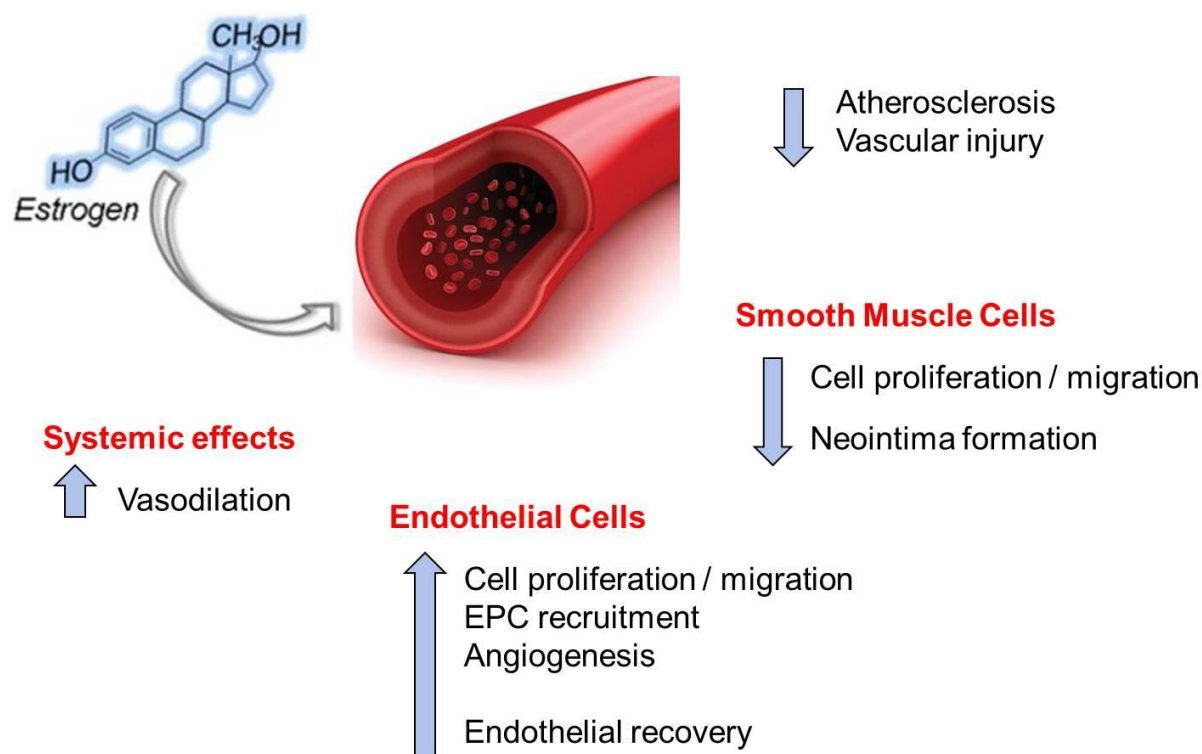
## 1.4 The effects of Estrogens on the cardiovascular system

There are several risk factors for CVD: hypertension, diabetes, high cholesterol, obesity, inappropriate lifestyle, high age and male gender. Epidemiologic studies provide evidence that the incidence of CVD is generally higher in men, compared to age-matched women [108, 109], and in postmenopausal compared to premenopausal women. Indeed, compared to premenopausal women, increased intimal thickening and plaque formation is documented in postmenopausal women [110]. These findings, and the fact that upon menopause (around the age of 55) the ovaries become dysfunctional leading to a drop in endogenous estrogen synthesis, suggest that decreased estrogen levels are associated with increased development of CVDs [111].

The association between vascular pathology and estrogen deficiency is further supported by the fact that postmenopausal women taking hormone replacement therapy (HRT) present a reduced risk of coronary disease [112, 113] and all-cause mortality [114, 115]. Even though several observational studies in humans and experimental studies in animals and isolated cells support the protective role of HRT in the cardiovascular system [115-117], the Women's Health Initiative (WHI) trial and the Heart and Estrogen/ Progestin Replacement Study (HERS) contradicted these findings [118, 119]. The reasons for the controversial role of HRT in the vascular system are not known, however, re-evaluation of the data from these studies revealed that the participant age and condition of the vessels might be critical. Indeed, HRT was effective in younger healthy women, as opposed to older participants with established vascular pathologies [120], suggesting that timing of HRT initiation is crucial for vaso-protection by estrogens [121, 122].

### 1.4.1 Beneficial actions of estradiol on vascular cells

*In vivo* studies provide strong evidence for the beneficial effects of E2, which has been shown to prevent pathological processes associated with the development of hypertension, thrombosis, restenosis, cardiomyopathy, atherosclerosis and other CVDs. Indeed, there is abundant evidence for an inhibitory role of E2 in vascular remodeling processes like neointimal thickening, cholesterol-induced atherosclerosis, injury-induced neointima formation and allograft-induced dysplasia [123-127].



**Figure 10:** Summary of the principal beneficial actions of E2 in the vascular system. Adapted from Menazza and Murphy [35]. *EPC, Endothelial Progenitor Cells.*

The anti-occlusive effects of E2 are mediated via multiple mechanisms. Both indirect systemic actions and direct regulation of vascular cell function [128, 129] are summarized in **Figure 10**. The main systemic actions include induction of vasodilation, through promotion of endothelial NO release and prostaglandin synthesis. Additionally, E2 reduces the production of vasoconstrictors such as angiotensin II, endothelin-1 and catecholamine [116]. Among the recognized effects of E2 on vascular cells, E2 promotes physiological functions in ECs by different mechanisms. First, it induces proliferation and migration of ECs *in vitro* [130] and promotes endothelial regeneration *in vivo* [131, 132]. Second, it contributes to the repair of the endothelium through the mobilization of EPC, inflammatory immune cells and platelets [133, 134]. Third, it stimulates angiogenesis by secreting important pro-angiogenic factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and nitric oxide (NO) [135, 136]. Since EC health is essential for vascular SMC quiescence, improvement of endothelial integrity can additionally contribute to the beneficial effects of E2. Endothelial damage or dysfunction results in SMC proliferation and migration and E2 has been proven to inhibit both these

processes *in vitro* as well as *in vivo* [124-127]. Anti-mitogenic effects of E2 result from the release of growth inhibitory molecules such as NO, cGMP and cAMP [137, 138].

The recognized effects of estrogens go beyond the mechanisms elucidated here and encompass multiple pathways and cell types contributing to the pathophysiology of CVD. [139] However, the key mechanisms to understand the protective actions of estrogens in the vascular system still need to be elucidated.

## **1.4.2 Molecular mechanisms of estradiol-mediated vascular protection**

### **1.4.2.1 Endothelial cells**

E2 induces regeneration and repair of the endothelium principally by stimulating vasculogenesis via different intracellular signaling pathways, including the PI3K/ Akt and VEGF pathway and the TGF $\beta$  signaling pathway ALK1/ SMAD1/5/8.

#### *PI3K/ Akt and VEGF*

The PI3K/ Akt pathway regulates multiple cellular functions including metabolism, growth, proliferation, migration, survival, transcription, protein synthesis and angiogenesis [140, 141]. This pathway is activated by growth factors such as VEGF, and several studies support its importance in tube formation and other processes associated with angiogenesis [142, 143] (**Figure 11A**). An important target of PI3K/ Akt signaling is eNOS, which generates NO upon phosphorylation, thus contributing to EC-induced vasodilation. NO in turn induces VEGF expression hence, NO and VEGF stimulate each other in a feedback circle until the PI3K signaling is terminated [144-146].

The role of VEGF in angiogenesis has been intensively studied and established [147]. VEGF is important in embryogenesis, as inactivation of one *VEGF* allele results in defective vascularization and embryonic lethality in mice [148, 149]. VEGF production is regulated in response to external stimuli by numerous cytokines and growth factors, which modulate VEGF expression to exert pro- or anti-angiogenic effects. As already described, among the molecules that up-regulate VEGF expression is NO, which contributes to VEGF-stimulated vasodilation [150]. Moreover, NO production is induced by VEGF through the PI3K/ Akt pathway, indicating that a positive feedback loop exists between these two factors.

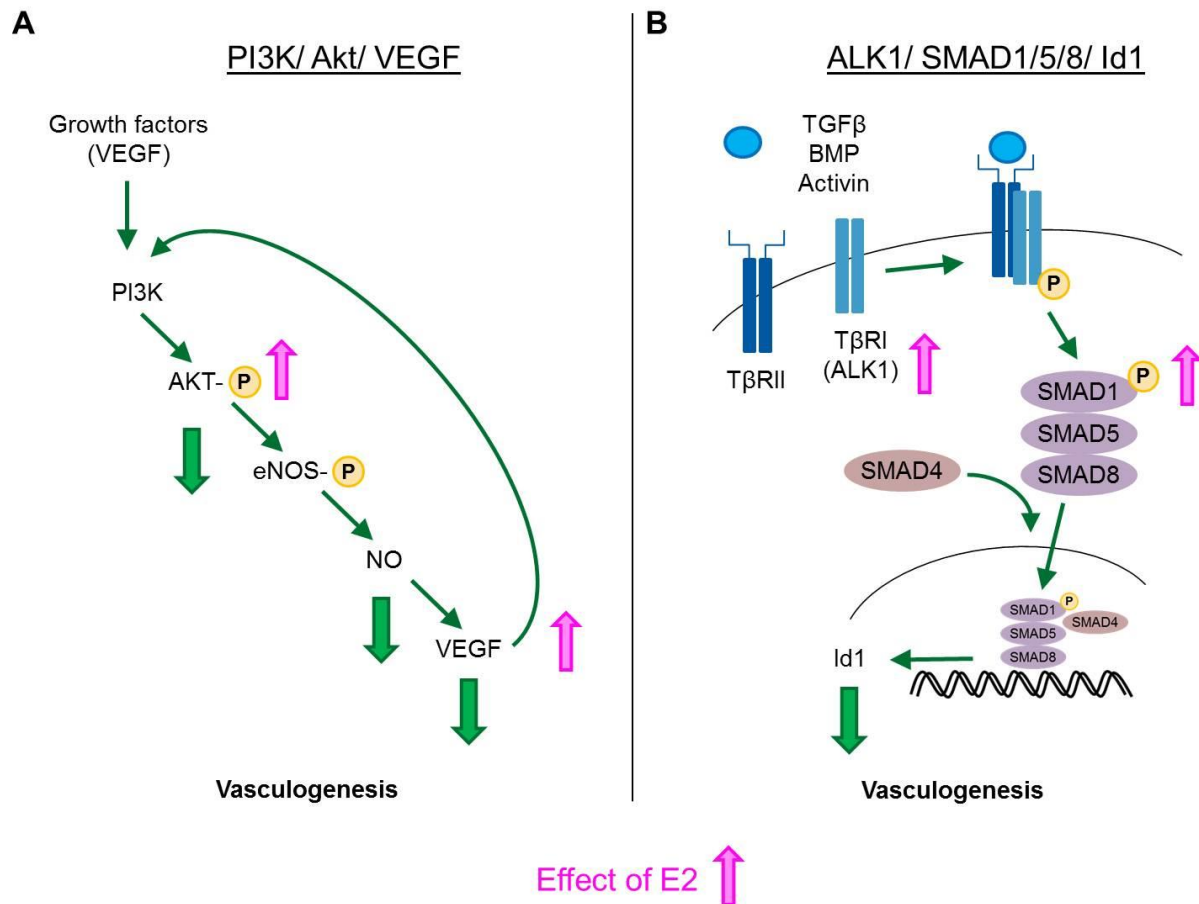
Several studies provide evidence that estrogens activate PI3K/ Akt signaling and that this pathway mediates the stimulatory actions of E2 on EC proliferation migration and tube formation [136, 151-153]. Moreover, there is evidence that E2 stimulates VEGF and NO [154], which mediate its angiogenic effects [135]. Since VEGF is both a ligand and a downstream product in the PI3K/ Akt pathway, it is not clear whether E2 activates the pathway by directly stimulating PI3K or via increased VEGF expression. However, these results indicate that PI3K/ Akt signaling and VEGF are important mediators of estrogen stimulated angiogenesis in ECs.

#### *ALK1/ SMAD1/5/8*

A second important pathway in the regulation of EC function is the ALK1/ SMAD1/5/8 pathway (**Figure 11B**). ALK1 is a receptor for some members of TGF $\beta$  superfamily. This family includes TGF $\beta$ , Bone Morphogenetic Proteins (BMPs), growth differentiation factors (GDFs), activins, inhibins and myostatins. The TGF $\beta$  signaling pathway plays an essential role in the regulation of multiple cellular functions such as growth, adhesion, migration, apoptosis and differentiation [155]. TGF $\beta$  signaling is complex, since it encompasses numerous ligands, receptors and their various combinations depending on the cell type and cellular context.

ALK1 is highly expressed in ECs and exerts its function via the activation of SMAD1/5/8. SMADs transduce extracellular signals from TGF $\beta$  ligands to the nucleus where they activate downstream gene transcription. Briefly, upon binding to the ligand a TGF $\beta$  type II receptor complexes with the type I receptors ALK1 or ALK5 and phosphorylates them. ALK1 phosphorylation leads to the recruitment and phosphorylation of SMAD1/5/8, which complexes with SMAD4 to translocate to the nucleus [156], where it induces the expression of Inhibitor of Differentiation (Id1), leading to increased migration, proliferation and tube formation of ECs [157-159]. Although ALK1 expression has been shown to correlate with increased proliferation, migration and tube formation [160-163], its role in ECs is controversial, since other studies have found inhibitory effects on the same processes [164, 165]. These opposing results might be due to the complexity of TGF $\beta$  signaling and differences in cell type and context [166]. The importance of ALK1/ SMAD1/5/8/ Id1 for EC functions is further supported by the fact that mutations in *ALK1* and in *SMAD* genes lead to severe defects in embryogenesis and early lethality due to vascular abnormalities in murine embryos [167, 168]. There is evidence that estrogens

regulate TGF $\beta$  signaling. Indeed, E2 inhibits the TGF $\beta$  pathway in MCF-7 breast cancer cells [169]. Moreover, previous studies from our laboratory demonstrate that E2 mediates vasculogenesis and endothelial growth via stimulation of ALK1/ SMAD1/5/8 signaling [170, 171].



**Figure 11:** Simplified schematic representation of the pro-vasculogenesis pathways PI3K/ Akt/ VEGF (**Panel A**) and ALK1/ SMAD1/5/8/ Id1 (**Panel B**) and their regulation by E2. *PI3K*, Phosphoinositide 3 Kinase; *Akt*, Protein Kinase B; *VEGF*, Vascular Endothelial Growth Factor; *eNOS*, endothelial Nitric Oxide Synthase; *NO*, Nitric Oxide; *ALK1*, Activin Receptor-Like Kinase 1; *TGF $\beta$* , Transforming Growth Factor  $\beta$ ; *BMP*, Bone Morphogenetic Protein; *T $\beta$ R*, TGF $\beta$  Receptor; *Id1*, Inhibitor of differentiation. Panel B adapted from Plutino [172].

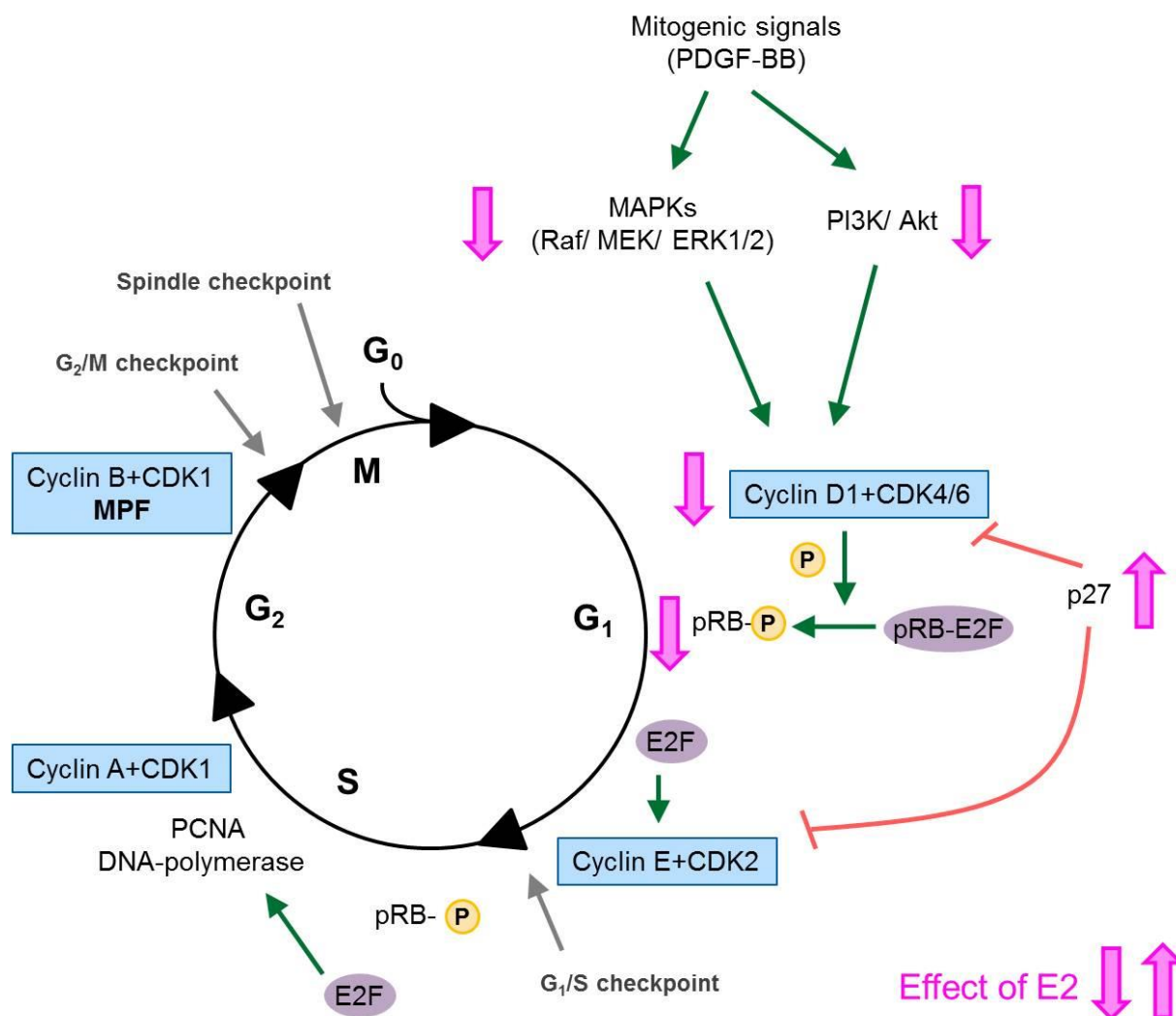
#### 1.4.2.2 Vascular smooth muscle cells

Abnormal growth of vascular SMCs significantly contributes to intimal thickening in atherosclerosis and is initiated by growth factors such as PDGF-BB, which are released as a result of vascular injury. Although the molecular mechanisms underlying the anti-mitogenic actions of estrogens in vascular SMC are far from clear,

current evidence indicates that they might regulate several key molecules required for S phase entry and cell cycle progression (**Figure 12**).

Upon stimulation, quiescent vascular SMCs begin to proliferate by entering the cell cycle in G<sub>1</sub>-phase and activating genes required for transition from G<sub>0</sub> to G<sub>1</sub> phase [173-175]. The accumulation of G<sub>1</sub> cyclins (cyclins D) and their association with Cyclin-Dependent Kinase (CDK)-4 and CDK6 leads to the hyperphosphorylation of Retinoblastoma protein (pRB). The activation of pRB is central in the G<sub>1</sub> to S phase transition of the cell cycle. In quiescent cells pRB is bound to and inhibits the transcription factor E2F. However, upon pRB phosphorylation by cyclin-CDK complexes, E2F is released and activates the transcription of genes necessary for G<sub>1</sub>/S transition, such as the genes encoding cyclin E and CDK2, and proteins required for DNA synthesis in S-phase, including the Proliferating Cell Nuclear Antigen (PCNA) and the DNA-polymerase [176, 177]. After replication of the genomic material, the cells proceed into G<sub>2</sub> phase and undergo cellular division. Estrogens have been shown to inhibit PDGF-BB-induced [<sup>3</sup>H]-thymidine incorporation [178, 179] and to reduce pRB phosphorylation and cyclin D1 expression in vascular SMCs [180]. Moreover, there is evidence that E2 prevents degradation of the CDK inhibitor p27 in pulmonary artery SMCs [181], indicating that its anti-mitogenic actions are in part due to the regulation of G<sub>1</sub>/S-phase cell cycle proteins.

PDGF-BB and other growth factors regulate cellular activities ranging from gene expression, proliferation, migration, metabolism and apoptosis, through the activation of various signaling cascades. The Raf/ MEK/ ERK1/2 pathway has been shown to be stimulated after arterial injury [182, 183], and to play a role in vascular SMC proliferation [184] and migration [185]. A second pathway activated by vascular injury is the PI3K/ Akt pathway [186, 187], which is also implicated in promoting cell cycle progression [188]. PI3K is required for mitogen-induced cyclin D1 expression and p27 reduction and delays G<sub>1</sub>/S exit [189]. Moreover, there is evidence for Akt regulation of Raf, suggesting a crosstalk between the PI3K/ Akt and the MAPK pathways [190]. E2 inhibits ERK1/2 activity [178] and has been associated to PI3K signaling [191], indicating that estrogens not only inhibit cell cycle progression, but also play a role in the regulation of upstream signaling cascade.

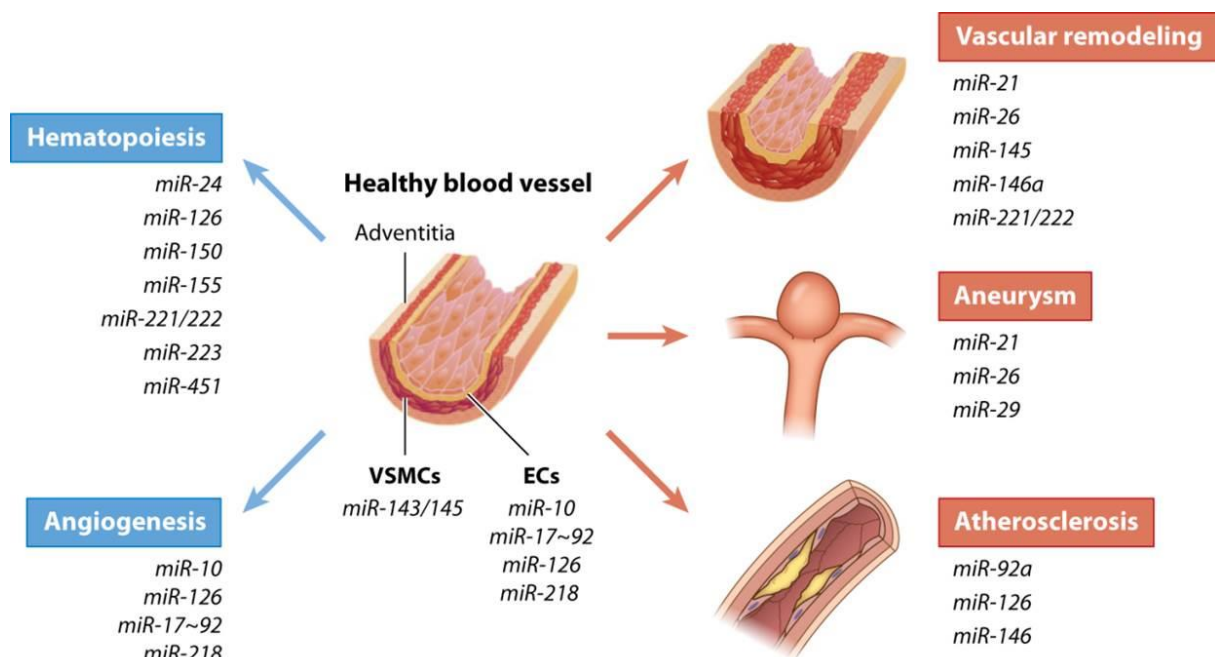


**Figure 12:** Simplified schematic representation of the cell cycle in vascular SMCs and its regulation by E2. *G<sub>0</sub>*, quiescence; *G<sub>1</sub>*, Gap 1; *S*, Synthesis; *G<sub>2</sub>*, Gap 2; *M*, Mitosis; *PDGF-BB*, Platelet-Derived Growth Factor BB; *MAPK*, Mitogen Activated Protein Kinase; *PI3K*, Phosphoinositide 3-Kinase; *CDK*, Cyclin-Dependent Kinase; *RB*, Retinoblastoma protein; *PCNA*, Proliferating Cell Nuclear Antigen; *MPF*, Mitosis Promoting Factor. Adapted from Barchiesi [192].



## 1.5 MicroRNAs in cardiovascular biology and disease

The importance of miRNAs in many clinically relevant biological and pathophysiological processes, including those of the cardiovascular system, has been demonstrated in multiple experimental and clinical studies [193-195]. General ablation of miRNAs in the cardiovascular system through tissue-specific deletion of Drosha, DGCR8, AGO2 or Dicer provided evidence for their critical roles in cardiovascular development and function [196, 197]. For instance, miRNAs are involved in the regulation of embryonic heart development [198, 199] and cardiomyocyte differentiation [200-202]. Further, several miRNAs have been reported to be essential in hematopoiesis [203] and angiogenesis [204] and there is evidence about miRNAs necessary for vascular SMC growth and differentiation [205], implying that miRNAs play a crucial role for the physiological function of both ECs and vascular SMCs.



**Figure 13:** Role of miRNAs in healthy and diseased vasculature. Left: examples of miRNAs important for hematopoiesis and angiogenesis. Center: cell type specific miRNAs in vascular SMCs and ECs. Right: examples of miRNAs aberrantly expressed after pathological changes. Depicted from Hata [193].

Given the importance of miRNAs in the vascular system, it is not surprising that their dysregulation is associated with pathological conditions such as heart disease, ischemia, tumor angiogenesis, atherosclerosis and restenosis [206]. Moreover, many

*in vitro* and *in vivo* studies provide evidence that vascular remodeling, the underlying mechanisms for atherosclerosis and most of the vascular diseases, develops as a result from aberrant expression of miRNAs in ECs and vascular SMCs [207] (**Figure 13**).

### 1.5.1 MicroRNAs in endothelial cells

EC proliferation, migration and angiogenesis are important biological processes for the recovery of injured or dysfunctional endothelium, the primary process of vascular remodeling. Genetic deletion of Dicer in mice resulted in abnormal blood vessel formation and embryonic lethality [204], and siRNA-mediated knock-down of Dicer in ECs resulted in a failure of capillary sprouting and tube formation [208, 209], revealing a role for miRNAs in the regulation of EC function. Individual miRNAs have since been shown to play positive and negative regulatory roles in angiogenesis.

Among the positive regulators of EC function, the cell specific miR-126 promotes angiogenesis and vascular integrity by repressing negative regulators of the VEGF signaling pathway [210], and its deletion leads to vascular abnormalities and endothelial leakage [211]. Another example of a miRNA regulating EC function is the hypoxia-induced miR-210, which stimulates EC proliferation and migration, thus enhancing angiogenesis in response to low oxygen levels [212, 213]. Members of the miR-17-92 cluster, which includes miR-17, -18a, -19a/b, -20a, and miR-92a, have been shown to influence angiogenesis in either positive or negative ways, depending on the cellular context. A negative regulator of vessel growth is miR-92a, which is up-regulated after ischemia. Knock-down of miR-92a *in vivo* improved recovery from ischemic damage due to enhanced angiogenesis [214]. Moreover, studies using HUVECs demonstrated that miR-221 and miR-222 decrease cell survival, migration and endothelial tube formation [215].

### 1.5.2 MicroRNAs in smooth muscle cells

In healthy normal blood vessels, vascular SMCs are differentiated cells with a contractile phenotype. However, damage of the vascular system triggers vascular SMC state alteration to a synthetic pro-proliferative state. They then migrate into the subendothelial space, leading to neointimal thickening and potential vessel occlusion. Several studies provide evidence for the role of miRNAs in vascular SMC phenotypic modulation. The vascular SMC specific miR-143/miR-145 cluster is necessary for

SMC contractility and the maintenance of a quiescent cell phenotype [71, 216]. Vessels of knock-out mice are thin and distended and display a decreased vascular tone [217]. In addition, the abrogation of miR-143/miR-145 *in vitro* reduced actin stress fiber formation, resulting in de-differentiation and acquirement of a pro-proliferative phenotype [218]. Further, expression of miR-143/miR-145 gene cluster is down-regulated in the carotid artery after mechanical injury and its overexpression blunted the change in phenotype and reduced injury-induced neointima formation [219]. In contrast, miR-21, which is increased after injury, promoted vascular SMC proliferation *in vitro* and induced neointima formation in response to balloon angioplasty of the carotid artery *in vivo* [220]. Another miRNA positively regulating the switch to a less contractile phenotype of vascular SMCs is the PDGF-BB-induced miR-221, which has been demonstrated to mediate the pro-mitogenic effects of PDGF-BB by blocking the expression of contractile genes and stimulating cell de-differentiation, proliferation and migration [221]. Knock-down of miR-221 reduced vascular SMC proliferation *in vitro* and inhibited injury-induced neointima formation in rat carotid artery [222]. Besides, miR-221 was reported to be up-regulated in quiescent cells in response to vascular injury and in proliferative vascular SMCs [223].

### **1.5.3 MicroRNA cell-specific effects**

Recent studies in cancer cells provide evidence of miRNA cell specific effects, for instance miR-21 increased HeLa cell apoptosis, meanwhile inhibiting glioblastoma cell apoptosis [224, 225]. These cell specific roles of miRNAs appear to also be relevant in the vascular system, where miRNAs have been demonstrated to differentially regulate EC and SMC functions.

A first example is miR-21, which was previously described to be a pro-proliferative miRNA in vascular SMCs [220]. In contrast, miR-21 was demonstrated to reduce EC and EPC proliferation and was anti-angiogenic in ECs [226, 227]. Another miRNA with a biphasic effect in vascular cells is miR-26a. Evidence indicates that miR-26a inhibits EC function [228], although it suppresses vascular SMC differentiation and promotes cell growth [229]. Lastly, the cell specific effects of miR-221 were compared in one study showing that miR-221 actively contributes to pathological vascular remodeling by reducing EC survival, growth and tube formation ability, while promoting SMC proliferation and migration [230].



## 2 General Hypothesis and Objectives

CVD is the leading cause of mortality in women, and endogenous estrogens as well as estrogen replacement therapy have shown to induce cardiovascular protection. Experimental observations from animal studies provide evidence for the vaso-protective actions of estrogens [109, 111-114]. These are further supported by clinical studies in women in early menopause receiving estrogen therapy [118-122]. Although estrogens are known to protect against vaso-occlusive disorders, the mechanism(s) by which they induce their protective effects are still unclear. Endothelial dysfunction and damage are key processes, which contribute to abnormal vasodilation and growth of SMCs resulting in vaso-occlusive disorders. Treatment with E2 has been shown to improve endothelial dependent relaxation and promote endothelial regeneration by inducing EC growth, recruitment of EPCs and stimulation of angiogenesis [131, 132, 135, 136]. Furthermore, E2 inhibits SMC proliferation and migration, thus preventing vascular remodeling processes leading to vascular occlusion [125-127]. Recent studies suggest that the differential effects of E2 on ECs and SMCs are potentially mediated via mechanisms linked to the estrogen receptors ER $\alpha$ , ER $\beta$  and GPER [35-37] or to ER-independent pathways involving downstream metabolites of E2 [14, 15]. Recent studies provide evidence that miRNAs mediate the effects of growth factors. However, the role and contribution of non-coding miRNAs in mediating the growth effects of estrogen on vascular cells remain largely undefined. Since miRNAs actively participate in the processes associated with vascular remodeling [193-195] and are known to mediate the biologic actions of E2 in several cancer cells [88], it is feasible that the protective actions of E2 on the vascular system are potentially mediated via the modulation of miRNA expression. Indeed, up-regulation of miR-203 by E2 is reported to contribute to the anti-mitogenic effects in vascular SMCs [94]. Moreover, cell specific effects of miRNAs have been observed in many tissues including the vascular system. For example, miR-221 actively contributes to injury-induced neointima formation by inhibiting EC growth and promoting SMC growth [222]. This biphasic effect of miR-221 mimics the differential actions of E2 on vascular cells, which specifically promotes EC repair/ recovery and inhibit SMC growth. Hence, it is feasible that the beneficial effects of E2 on the cardiovascular system are due to the modulation of cell specific miRNAs, such as miR-221.

**Based on the above findings, we hypothesize that E2-regulated miRNAs participate in mediating the protective actions of E2 on the vascular system and are responsible for the differential effects on EC and vascular SMC growth.**

The **first aim** of this study was to **identify E2-regulated miRNAs in ECs and SMCs**. To achieve this, we used data from preliminary small miRNA sequencing in EPCs to screen for differentially expressed miRNAs after E2 treatment and subsequently validated the candidate miRNAs by RT-qPCR in HUVECs and HCASMCs.

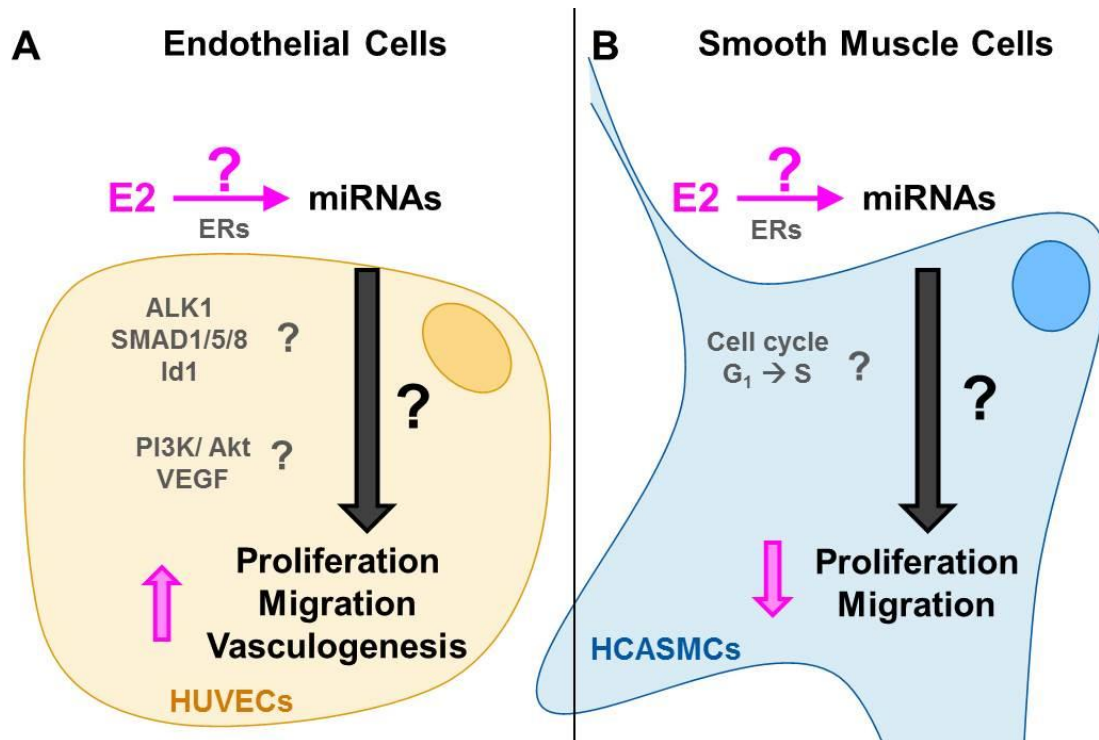
The **second aim** was to **investigate the role of the selected miRNAs on both HUVEC and HCASMC cell function**. To accomplish this objective, we assessed proliferation, migration, vasculogenesis and viability of HUVECs as well as proliferation, migration and viability of HCASMCs in response to miRNA modulation with specific miRNA mimics and antimirs. Moreover, we examined the response of miRNA modulation after E2 treatment, to **assess whether the selected miRNAs mediate the differential effects of E2 in these cells**.

The **third aim** was to **examine the potential intracellular mechanisms of miRNA-mediated vasculogenesis and proliferation in HUVECs and in HCASMCs, respectively**. Hence we investigated and identified molecular mechanisms, which are either involved in E2-induced vasculogenesis in HUVECs or participate in the anti-mitogenic action of E2 in HCASMCs. E2 has been shown to stimulate vasculogenesis via the PI3K/ Akt pathway [152] and via stimulation of VEGF-A [231]. Additionally, the ALK1/ SMAD1/5/8 pathway plays a key role in embryonic angiogenesis [167, 168, 232]. Therefore, we **investigated the role of the selected miRNA on the PI3K/ Akt pathway, the ALK1/ SMAD1/5/8 /Id1 pathway and VEGF-A in HUVECs**. E2 inhibits SMC proliferation partly via the prevention of cell cycle progression from G<sub>1</sub> to S phase. Therefore, we **studied the role of the selected miRNAs on the regulation of several early cell cycle proteins in HCASMCs**.

The role of the classic estrogen receptors, ER $\alpha$ , ER $\beta$ , and the recently discovered GPER, in mediating the beneficial effects of E2 in vascular cells is well established [233]. Therefore, our **forth aim** was to **identify the receptor involved in the modulation of the E2-regulated miRNAs**. To accomplish this, we assessed the expression levels of the miRNAs in response to specific ER agonists and antagonist.

The regulation of mature miRNA expression potentially occurs at the transcriptional level or during processing of pri-miRNAs and pre-miRNAs to functionally active mature miRNAs [86]. Since ERs are transcription factors and regulate gene expression, the **fifth aim** was to **elucidate whether E2 regulates the transcription of the selected miRNAs**. To assess this, we analyzed the expression level of pri-miRNA following E2 treatment.

**Overall, the main goal of this study was to investigate the role of miRNAs in mediating the growth effects of E2 in vascular ECs and SMCs. The results will help to improve our understanding about the mechanism(s) by which E2 regulates growth and function of vascular cells that actively participate in vascular remodeling processes. Moreover, these findings would help to develop better (miRNA-based) therapy for menopause associated CVD.**



**Figure 14: Schematic representation of hypotheses and objectives.** The main purpose of the study was to identify E2-regulated miRNAs, which contribute to the differential actions of E2 in vascular cells. **Panel A:** We used HUVECs to assess the role of the selected miRNAs in EC function and on the possible molecular mechanisms involved. **Panel B:** We used HCASMCs to assess the role of the selected miRNAs in vascular SMC growth and on the possible molecular mechanisms involved. *HUVEC*, Human Umbilical Vein Endothelial Cell; *HCASMC*, Human Coronary Artery Smooth Muscle Cell; *E2*, 17 $\beta$ -estradiol; *ER*, Estrogen Receptor; *ALK1*, Activin Receptor-Like Kinase 1; *Id1*, Inhibitor of Differentiation; *PI3K*, Phosphoinositide 3 Kinase; *Akt*, Protein Kinase B; *VEGF*, Vascular Endothelial Growth Factor; *G<sub>1</sub>*, Gap1; *S*, Synthesis.





### 3 Materials

#### 3.1 Cell culture

EPC	Lonza, MD, USA (00189423)
HCASMC	Life Technologies, CA, USA (C-017-5C)
HUVEC	Lonza, MD, USA (CC-2517)
2-Propanol	Kantonsapotheke, Zürich, CH
Antibioticum-Antimycotium (AA)	Life Technologies, CA, USA (15240-096)
Bovine Serum Albumin (BSA)	Sigma-Aldrich, MO, USA (T-9647)
Collagen, Rat Tail	Roche, Mannheim, DE (11179179001)
Dulbecco's Modified Eagle Medium (DMEM)-F12	Sigma-Aldrich, MO, USA (D-6434)
Endothelial Basal Medium (EBM)-2	Lonza, MD, USA (CC-3156)
EGM-2 SingleQuots	Lonza, MD, USA (CC-4176)
Fetal Calf Serum (FCS)	Thermoscientific, MA, USA (SH30070)
FCS Charcoal Stripped	Thermoscientific, MA, USA (SH3006803)
Hank's Buffered Salt Solution (HBSS)	Biocencept, Allschwill, CH (3-02K34-I)
L-Glutamine	Life Technologies, CA, USA (25030-024)
Low Serum Growth Supplements (LSGS)	Life Technologies, CA, USA (S003-10)
M231 media	Life Technologies, CA, USA (M-231-500)
Phosphate Buffered Saline (PBS)	Life Technologies, CA, USA (18912-014)
Smooth Muscle Growth Supplements (SMGS)	Life Technologies, CA, USA (S-007-25)
Trypsin	Sigma-Aldrich, MO, USA (T-3924)

#### 3.2 Chemicals and buffers

Acetic acid	Merck, Darmstadt, DE (100063)
ALK1 Fc Chimera, Human Recombinant	R&D Systems, MN, USA (370-AL-100)
Ammonium Persulfate (APS)	Sigma-Aldrich, MO, USA (A-3678)
Aprotinin	Sigma-Aldrich, MO, USA (A-1153)
Bichinonic Assay (BCA) Protein Assay Kit	Pierce, USA (23227)
Bovine Serum Albumin (BSA)	Sigma-Aldrich, MO, USA (A-3059)
Cell Lysis Buffer	Cell Signaling, Danvers, USA (9803)

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Cell proliferation ELISA, BrdU	Roche, Basel, CH (11669915001)
Chloroform	Sigma-Aldrich, MO, USA (C-2432)
Coulter Clenz cleaning agent	Kantonsapotheke, Zürich, CH
Coulter Isoton II diluent	Kantonsapotheke, Zürich, CH
Diethylpyrocarbonate (DEPC)-treated water	Life Technologies, CA, USA (AM9916)
DNA Gel loading dye (6x)	Thermoscientific, MA, USA (R0611)
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, MO, USA (D-2650)
Dithiotheritol (DTT)	Fermentas, MD, USA (R-0891)
DPN	Tocris, Bristol, UK (1494)
E2	Steraloids, RI, USA (E950)
Ethanol (EtOH), abs	Kantonsapotheke Zurich, CH
Fibrinogen Type I	Sigma-Aldrich, MO, USA (F-0895)
G1	Calbiochem, Darmstadt, DE (371705)
Glucose	Sigma-Aldrich, St.Louis, USA (G-6152)
Guava EasyCheck Kit	Millipore, Darmstadt, DE (4500-0025)
Guava Instrument Clean Fluid	Millipore, Darmstadt, DE (4200-0140)
Hoechst33342	Life Technologies, CA, USA (H3570)
Hydrochloric acid (HCl)	Merck, Darmstadt, DE (109973)
Hyperfilm ECL	Amersham, CH (RPN2103K)
ICI 182-780	Tocris, Bristol, UK(1047)
Laemmli Sample Buffer	Biorad, Reinach CH (161-0747)
Lipofectamine2000	Life Technologies, CA, USA (11668019)
LY0294002	Merck, Darmstadt, DE (440202)
Magnesium Chloride (MgCl <sub>2</sub> )	Sigma-Aldrich, MO, USA (M-2393)
Matrigel	BD Biosciences, NJ, USA (356237)
Methanol	Sigma-Aldrich, MO, USA (32213)
miRNeasy Mini Kit	Qiagen, CA, USA (217184)
MPP	Tocris, Bristol, UK (1991)
NewBlot Nitro Stripping buffer	LI-COR, NE, USA (928-40030)
Nonfat dry milk	COOP, CH
PBS tablets	Life Technologies, CA, USA (18912-014)
PDGF-BB	Sigma-Aldrich, MO, USA (P-3201)
Phenyl-Methyl-Sulfonyl Fluoride (PMSF)	Sigma-Aldrich, MO, USA (P-7626)
Ponceau S Solution (2%)	Sigma-Aldrich, MO, USA (P-7767)

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Potassium Chloride (KCl)	Sigma-Aldrich, MO, USA (P-5405)
PPT	Tocris, Bristol, UK (1426)
Precision Plus Dual Color Standard	Biorad, Reinach CH (161-0374)
Propidium Iodide (PI)	Sigma-Aldrich, MO, USA (P-4170)
Quick-RNA MiniPrep Kit	Zymo Research, USA (R1055)
RNase A	Roche, Basel, CH (10109142001)
RNase Zap	Life Technologies, CA, USA (AM9782)
Rotiphorese Gel 30	Carl Roth GmbH, Karlsruhe, DE (3029.1)
Sodium Chloride (NaCl)	Sigma-Aldrich, MO, USA (71381)
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich, MO, USA (L-5750)
Sodium Hydroxide (NaOH)	Sigma-Aldrich, MO, USA (S-8045)
SuperSignal West Dura	Pierce, Rockford, USA (34076)
SuperSignal West Pico	Pierce, Rockford, USA (34077)
TaqMan Fast Advanced Master mix	Life Technologies, CA, USA (4444964)
TaqMan High-Capacity cDNA Reverse Transcription Kit	Life Technologies, CA, USA (4368814)
TaqMan miRNA Reverse Transcription Kit	Life Technologies, CA, USA (4366597)
Temed	Sigma-Aldrich, MO, USA (T-9281)
TGFβ1, Human Recombinant	R&D Systems, MN, USA (240-B)
Thiazol Blue Tetrazolium Bromide (MTT)	Sigma-Aldrich, MO, USA (M-5655)
Tris/Boric Acid/EDTA Buffer (TBE, 10x)	Bio-Rad, Reinach, CH (161-0770)
Tris/Glycine Buffer (TG, 10x)	Bio-Rad, Reinach, CH (161-0771)
Tris/Glycine/SDS Buffer (TGS, 10x)	Bio-Rad, Reinach, CH (161-0772)
Tris/HCl, 0.5M, pH 6.8	Biorad, Reinach, CH (161-0799)
Tris/HCl, 1.5M, pH 8.8	Biorad, Reinach, CH (161-0799)
Tween 20	Sigma-Aldrich, MO, USA (P-7949)
VEGF antibody, human	R&D Systems, MN, USA (MAB293)
VEGF	Sigma-Aldrich, MO, USA (V-7295)

### 3.3 MicroRNA sequences and primers

#### 3.3.1 Mature microRNA Sequences

hsa-miR-100	5' -	AACCCGUAGAUCCGAACUUGUG - 3'
hsa-miR-146a	5' -	UGAGAACUGAAUUCUUGGGUU - 3'
hsa-miR-193a-3p	5' -	AACUGGCCUACAAAGUCCCAGU - 3'
hsa-miR-203	5' -	GUGAAAUGUUUAGGACCACUAG - 3'
hsa-miR-221-3p	5' -	AGCUACAUUGUCUGCUGGGUUUC - 3'
hsa-miR-222	5' -	AGCUACAUCUGGCUACUGGGU - 3'
hsa-miR-638	5' -	AGGGAUCGCGGGCGGGUGGCGGCCU - 3'
RNU48	5' -	GATGACCCCAGGTAAGTCTGAGTGTGTCG CTGATGCCATCACCGCAGCGCTCTGACC - 3'
RNU49	5' -	CACTAATAGGAAGTGCCGTCAGAAGCGA TAACTGACGAAGACTACTCCTGTCTGATT - 3'

#### 3.3.2 Mimics and Antimirs

AccuTarget Human miRNA Antimirs	Bioneer, CA, USA (SMI-001)
AccuTarget Human miRNA Mimics	Bioneer, CA, USA (SMM-001)
AccuTarget miRNA Antimir Negative Control #1	Bioneer, CA, USA (SMC-2101)
AccuTarget miRNA Mimic Negative Control #1	Bioneer, CA, USA (SMC-2001)
AccuTarget miRNA Mimic Positive Control	Bioneer, CA, USA (SMC-1001)
miRIDIAN miRNA Antimir Control with Dy547	
	Dharmacon, CO, USA (IP-004500-01-05)
miRIDIAN miRNA Mimic Control with Dy547	
	Dharmacon, CO, USA (CP-004500-01-05)

#### 3.3.3 RT-qPCR primers

TaqMan miRNA assays	Life Technologies, CA, USA (4427975)
hsa-miR-100	Assay ID 000437
hsa-miR-146a	Assay ID 000468
hsa-miR-193a-3p	Assay ID 002250
hsa-miR-203	Assay ID 000507
hsa-miR-221-3p	Assay ID 000524
hsa-miR-222	Assay ID 002276

hsa-miR-638	Assay ID 001582
RNU48	Assay ID 001006
RNU49	Assay ID 001005
TaqMan Pri-miRNA Assays	
Life Technologies, CA, USA (4427012)	
hsa-pri-miR-193a	Assay ID Hs03303307_pri
hsa-pri-miR-221	Assay ID Hs03303007_pri
TaqMan Gene Expression Assays	
Life Technologies, CA, USA (4331182)	
GAPDH	Assay ID Hs99999905_m1
hPRT1	Assay ID Hs99999909_m1

### 3.4 Antibodies

#### 3.4.1 Primary antibodies

Anti- $\beta$ -Actin	Sigma-Aldrich, MO, USA (A-5441)
Anti-Akt	Cell Signaling Tech., MA, USA (9272)
Anti-phospho-Akt (Ser473)	Cell Signaling Tech., MA, USA (9271)
Anti-ALK1	Santa Cruz, TX, USA (sc-28976)
Anti-CDK4	Millipore, CA, USA (07-659)
Anti-Cyclin D1	Millipore, CA, USA (06-137)
Anti-Cyclin E	Cell Signaling Tech., MA, USA (4129)
Anti-GAPDH	Abcam, Cambridge, UK (ab9484)
Anti-I $\delta$ 1	Santa Cruz, TX, USA (sc-488)
Anti-p27	Millipore, CA, USA (04-240)
Anti-PCNA	Millipore, CA, USA (CBL407)
Anti-pRB	BD Biosciences, NJ, USA (554136)
Anti-VEGF A	Santa Cruz, TX, USA (sc-152)

#### 3.4.2 Secondary antibodies

IGF-Peroxidase Conj. Goat anti-Mouse	Pierce, Rockford, USA (31430)
IGF-Peroxidase Conj. Goat anti-Rabbit	Pierce, Rockford, USA (31460)
IRDye 680 Conj. Goat anti-Mouse IgG	LI-COR, NE, USA (926-32220)
IRDye 800 Conj. Goat anti-Rabbit IgG	LI-COR, NE, USA (926-32211)
IRDye 680 Conj. Goat anti-Rabbit IgG	LI-COR, NE, USA (926-68021)
IRDye 800 Conj. Goat anti-Mouse IgG	LI-COR, NE, USA (926-68020)

### 3.5 Plastic material

μ-slides	ibidi, Planegg-Martinsried, DE (81506)
25-gauge needle	Terumo, Somerset, NJ, USA
Cell culture dishes, multiwell plates	Falcon by Beckton Dickinson
Cell culture flask	TPP, Trasadingen, CH
Cell lifter	Corninc Inc., NY, USA
Cryotubes	Nalge Nunc International, USA (377224)
Freezing container, Mr.Frosty	Nalgene Nunc International, USA
Nitrocellulose membrane	Whatman GmbH, Dassel, DE
Reaction tubes (15, 50 ml)	Falcon by Beckton Dickinson
Tips, Combitips	Eppendorf, Hamburg, DE
Reaction tubes (0.5, 1.5 ml)	Eppendorf, Hamburg, DE
PCR plates	Biorad, Reinach, CH

### 3.6 Instruments and software

#### 3.6.1 Western blot apparatus

Mini-PROTEAN 3 Electrophoresis Cell	Biorad, Reinach, CH
Mini-Trans-Blot Electrophoretic Transfer Cell	Biorad, Reinach, CH
Power Pac 200, Power Supplier	Biorad, Reinach, CH
X-ray cassette 13x18	Universitätsspital Zürich, CH

#### 3.6.2 Instruments

5417R Centrifuge	Eppendorf, Hamburg, DE
CFX Real-Time PCR Detection System	Biorad, Reinach, CH
Coulter Z1, Cell Counter	Coulter Electronics, Luton, UK
GUAVA easyCyte HT Flow Cytometer	Millipore, Darmstadt, DE
H54AR Precision balance	Mettler-Toledo, USA
Hera Cell 150, Cell incubator	Thermoscientific, MA, USA
Odyssey 3.0 Infrared Imaging System	LI-COR, NE, USA
C-DiGit Blot Scanner	LI-COR, NE, USA
Microtiter Centrifuge	Hettich, Bäch, CH
NanoDrop 1000	Thermoscientific, MA, USA
Olympus BX61 Microscope, Bright field	Olympus, Volketswil, CH

Olympus IX81 Microscope, Fluorescence	Olympus, Volketswil, CH
Rotina 46R Centrifuge	Hettich, Bäch, CH
Sonicator	Bandelin electronics, Berlin, DE
Tecan Infinite series M200 microplate reader	Tecan, Salzburg, AU
Tecan Infinite series M200 NanoQuant	Tecan, Salzburg, AU
X-ray Film developer	

### **3.6.3 Software**

Xcellence pro	Olympus, Volketswil, CH
Image Studio Lite	LI-COR, NE, USA
Magellan 6 and iControl	Tecan, Salzburg, AU
StatView, Version 5.0.1	SAS Institute, Cary, USA
GuavaSoft and InCyte	Merck Millipore, Darmstadt, DE
NanoDrop 1000	Thermoscientific, MA, USA
CFX Manager	Biorad, Reinach, CH
ImageJ	





## 4 Methods

### 4.1 Cell culture

**HCASMCs:** Human Coronary Artery Smooth Muscle Cells between 4-8th passage were cultured in 75 cm<sup>2</sup> flasks under standard tissue culture conditions (37°C, 5% CO<sub>2</sub>) in complete growing media: M231 culture medium supplemented with antibiotic-antimycotic (AA; 100 µg/ml streptomycin, 100 µg/ml penicillin and 0.025 µg/ml amphotericin B) and SMGS (5% v/v FCS, 2 ng/ml human basic Fibroblast Growth Factor, 0.5 ng/ml human Epidermal Growth Factor, 5 ng/ml Heparin, 5 µg/ml insulin and 0.2 µg/ml BSA). Medium was changed every two days until subconfluency. To split the culture, the cells were washed twice with HBSS (w/o Ca<sup>2+</sup> and Mg<sup>2+</sup>) and detached through trypsinization with 0.25% trypsin diluted in HBSS (w/o Ca<sup>2+</sup> and Mg<sup>2+</sup>). After 3 minutes incubation, an equal volume of growing medium was added and the cells were either diluted 4 times in 75 cm<sup>2</sup> flasks or plated on culture dishes or well plates. For experiments, HCASMCs were starved overnight and treated in HCASMCs starving media (M231, supplemented with AA).

**HUVECs:** Human Umbilical Vein Endothelial Cells between 4-12th passage were cultured in 75 cm<sup>2</sup> flasks under standard tissue culture conditions in complete growing media: DMEM-F12 supplemented with L-Glutamine, AA (100 µg/ml streptomycin, 100 µg/ml penicillin and 0.025 µg/ml amphotericin B), LSGS (2% v/v FCS, 1 µg/ml hydrocortisone, 10 ng/ml human Epidermal Growth Factor, 3ng/ml human basic Fibroblast Growth Factor, 10 µg/ml heparin) and 10% FCS. Medium was changed every two days and when the cells reached subconfluency they were washed twice with HBSS (w/o Ca<sup>2+</sup> and Mg<sup>2+</sup>) and trypsinized in 0.25% trypsin. After 2 minutes, double volume of growing medium was added to stop the detaching reaction. The cells were pelleted by centrifugation (5 minutes, 1200 rpm, room temperature) and the supernatant discarded prior resuspension of the cells in growing media and seeding 1:4 in 75 cm<sup>2</sup> flasks, culture dishes or well plates. For experiments, HUVECs were starved overnight and treated in HUVEC starving media (DMEM-F12, supplemented with L-Glutamine, AA and 1% BSA).

**EPCs:** Endothelial Progenitor cells between 4-8th passage were cultured under standard tissue culture conditions (5% CO<sub>2</sub> at 37°C). Prior cell seeding, 75 cm<sup>2</sup> flasks were coated with collagen (50 µg/ml in 0.02 N acetic acid). After 1 hr

incubation at room temperature, collagen was removed, the surface washed with PBS and the cells seeded in complete growing medium: EBM-2, supplemented with EGM-2 SingleQuots (containing human Epidermal Growth Factor, Hydrocortisone, human basic Fibroblast Growth Factor, VEGF, Insulin-like Growth Factor, Ascorbic Acid, Heparin and FCS), AA and 10% FCS. Medium was renewed every two days and upon confluency the cells were washed with HBSS (w/o  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ), trypsinized 2 minutes, neutralized in double volume of complete medium, centrifuged (5 minute, 1200 rpm, room temperature) and re-plated at 1:4 in tissue culture flasks, dishes or well-plates.

#### **4.1.1 Cryopreservation of cells**

Subconfluent cells were dislodged as described above. After trypsinization, fresh growing medium was added and the cells were centrifuged at 1200 rpm for 10 min at room temperature. The pellet was resuspended in ice-cold growing medium (4ml/ 75  $\text{cm}^2$  flask) containing 10% DMSO as cryoprotective agent. Aliquots of 1ml were gradually frozen to  $-70^\circ\text{C}$  in cryotubes in a Mr. Frosty box (freezing container filled with 2-propanol). For long-term storage the cells were kept in liquid nitrogen ( $-196^\circ\text{C}$ ).

#### **4.1.2 Cell defrosting**

Frozen cells in cryotubes were taken from liquid nitrogen and rapidly thawed under warm water. After disinfection of the vial, the content was slowly transferred to a 75  $\text{cm}^2$  culture flasks with 10 ml of the respective growing medium. The cells were incubated under standard tissue culture conditions (5%  $\text{CO}_2$  at  $37^\circ\text{C}$ ) and the media was replaced after 24 hr.

### **4.2 MicroRNA sequencing**

**EPCs** were seeded in 6 cm dishes and grown in complete media to 60% confluency prior 36 hr treatment with or without 10 nM E2 in triplicates in EBM-2 without phenol red and growth factors, supplemented with 2.5% steroid-free Charcoal Stripped FCS. RNA was extracted using the miRNeasy Mini Kit. First cell membranes and content were dissolved in 350  $\mu\text{l}$  lysis buffer. The collected lysates were passed through the columns to filter cell debris and the resulting solutions were mixed with 70% ethanol and transferred to RNA-binding columns. DNA was digested using DNases and

several washing steps were performed prior RNA elution with RNase-free water. The obtained RNA was stored at -80°C and handled over to the Functional Genomic Center Zürich for RNA quality and quantity assessment and the following sequencing. For the quality assessment Agilent Tape Station/ Bioanalyzer and Qubit fluorometer were used. For the sequencing, the libraries were prepared using Illumina's TruSeq Small RNA Library Prep Kit and sequenced on an Illumina HiSeq2000. The raw reads were first cleaned by removing adapter sequences using Trimmomatic [234]. Sequence alignment, quantification and quality control were performed using ncPRO (version 1.5.1) [235]. Bowtie was used for the mapping with ncPRO (with the following options '*-e 50 -a -m 50 --best --strata --nomaground*') and hg19 was used as genome reference for the mapping. The miRNA annotation is based on miRBase version 20. The detection of differentially expressed miRNAs was done using EdgeR [236].

### 4.3 Transfection with microRNA Mimics and Antimirs

**HCASMCs and HUVECs** were plated in the respective growth medium and allowed to recover at least for 24 hr. Before transfection, as described in **Table 1**, the appropriate amount of lipofectamine2000 was diluted in serum and antibiotic free DMEM-F12 in one tube (solution A). MiRNA Mimics or Antimirs (at a final concentration of 25 nM) were also diluted in serum and antibiotic free DMEM-F12 in a different tube (solution B). Both solutions were incubated 5 minutes at room temperature before being equally mixed (A+B) and incubated 20 minutes at room temperature to allow the miRNA oligonucleotides:lipofectamine2000 complexes to form. The cells were rinsed with serum and antibiotic free DMEM-F12 and the miRNA oligonucleotides:Lipofectamine2000 complexes added to each well.

	6 cm dish	6-well plate 3.5 cm dish	12-/ 24-well plate	96-well plate
<b>A</b> Lipofectamine 2000 Media	3 µl 147 µl	2 µl 98 µl	1 µl 49 µl	0.25 µl 25 µl
<b>B</b> Mimic / Antimir Media	0.9 µl 99.1 µl	0.6 µl 99.4 µl	0.3 µl 49.7 µl	0.075 µl 25 µl
Media on cells	1.5 ml	1 ml	0.5 ml	0.1 ml

**Table 1:** Transfection guideline for final 25 nM miRNA Mimics or Antimirs. The ratio of lipofectamine2000 and RNA were scaled for the different sizes of dishes or well plates.

After 5-6 hr the transfection media was removed and replaced by the regular growing media or respective starving media in presence or absence of the test agents. Mock transfection was carried out as described above but with the omission of the miRNA oligonucleotides.

### 4.3.1 Transfection efficiency

**HCASMCs and HUVECs** were transfected with a Dy547-labeled Mimic or Antimir and kept in the respective growing media for 24 hr before the transfection efficiency was assessed using fluorescence microscopy and flow cytometry. For the former, the cell nuclei were stained with Hoechst33342 (0.5 µg/ml) and incubated 30 minutes before microscope images were taken. For flow cytometry, the cells were detached by trypsinization, centrifuged and resuspended in 300 µl sample buffer (1 g/L glucose in PBS) before the percentage of fluorescent signal was measured by a GUAVA easyCyte Flow Cytometer. Cells transfected with miRNA negative Control Mimic or Antimir without a fluorescent tag were used as negative controls.

Efficient transfection with the Mimics was further proved using a positive Control Mimic specifically targeting GAPDH. Expression of GAPDH protein was assessed by Western blotting of samples at 24, 48 and 72 hr after transfection.

## 4.4 Cell proliferation studies

### 4.4.1 Cell counts

Cells were plated in 12-well plates and growth arrested in starving media for 24 hr, before stimulation with 10 nM E2 (**HUVEC**) or PDGF-BB (20 ng/ml) in presence or absence of E2 (**HCASMC**). Before transfection with Mimics and Antimirs, the cells were starved overnight and treated 5-6 hr post-transfection with or without PDGF-BB and E2. After 3 days, cells were dislodged by trypsinization, transferred into cuvettes and counted in a Coulter Counter.

### 4.4.2 DNA synthesis

**HCASMC** proliferation was additionally determined by a chemiluminescent BrdU incorporation ELISA kit (Roche). Cells were plated on a 96-well plate (5'000 cells per well) and allowed to attach overnight. The next day the cells were washed with PBS and incubated with or without PDGF-BB and E2 in starving media or transfected with

Mimics and Antimirs prior treatment. BrdU (10  $\mu$ M) was added after 24 hr and allowed to incorporate into the cells for further 24 hr. Cells were fixed and anti BrdU-antibody was added as described in the kit and the luminescent signal was measured using a Tecan Spectrofluorometer reader.

## 4.5 Migration studies

Cell migration was determined using a Scratch/ Wound Closure Assay. **HCASMCs** in 6-well plates and **HUVECs** in 12-well plates were grown to confluency before a scratch was made using a yellow pipet tip. The cells were then washed twice with PBS to remove cell debris and treated in presence or absence of the test agents. Cells transfected with miRNA Mimics and Antimirs were allowed to grow to confluency 48 hr post-transfection, before the scratch was made and the treatment added. Several images of the scratch were taken using the Olympus inverted microscope right after treatment (T0) and after 24 hr of incubation (T24). Wound closure was determined using Olympus Xcellence Pro software and calculated accordingly: (area T0- area T24)/ area T0.

## 4.6 Microvessel formation assay

**HUVECs** cultured in regular conditions and cells transfected with miRNA Mimics and Antimirs, kept 24 hr in growing media post-transfection, were collected by trypsinization, counted and resuspended in 1 ml per each treatment in DMEM-F12 supplemented with L-Glutamine, AA and 0.4% BSA at a density of 80'000 cells/ml in 15 ml falcon tubes. The cells were incubated with experimental agents 30 minutes prior transfer of 50  $\mu$ l (4'000 cells) to each well of the angiogenesis  $\mu$ -slide, previously coated with 10  $\mu$ l matrigel. The cells were allowed to form microvessels overnight and imaged with an Olympus inverted microscope. Five pictures were taken for each well at a 10x magnification. Microvessel length was determined using Olympus Xcellence Pro software.

## 4.7 Viability studies

### 4.7.1 MTT test

**HUVECs and HCASMCs** were plated in a 24 well plate and allowed to attach. After transfection the cells were kept in culture media or growth arrested for 48 hr. Subsequently the medium was replaced with fresh medium containing 0.5 mg/ml MTT. After additional incubation for 1 hr, the medium was aspirated and the cells lysed by the addition of 100  $\mu$ l DMSO. The absorbance was quantitated at 540 nm using a Tecan Spectrofluorometer reader.

### 4.7.2 PI staining

**HUVECs and HCASMCs** were plated in 6 cm dishes and allowed to attach. After transfection, the cells were kept in culture media for 24 hr prior collection through trypsinization followed by centrifugation (5 minutes, 1200 rpm, room temperature). The cells were then stained with PI (0.2  $\mu$ g/ml), incubated in the dark for 5 minutes and analyzed on a GUAVA easyCyte Flow Cytometer.

## 4.8 Protein expression analysis

**HCASMCs** were serum-starved overnight and treated 48 hr in presence or absence of PDGF-BB (20 ng/ml) and E2 (10-100 nM). Mimic and Antimir transfected cells were treated for 48 hr with the test agents post-transfection. **HUVECs** were serum-starved overnight and stimulated 45 minutes with 10 nM E2. Mimic and Antimir transfected cells were kept in growing media 48 hr post-transfection and stimulated 45 minutes with or without 10 nM E2. Cells were washed twice with HBSS and lysed by the addition of 60  $\mu$ l cell lysis buffer (containing 20 mM Tris pH7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2.5 mM sodium phosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM sodium vanadate, 0.5 PMSF and 0.2% SDS) followed by scraping. The samples were homogenized 2-10 seconds by sonication and stored at -20°C. The protein concentration was determined with the BCA Assay Kit according to the manufacturer's protocol. The absorbance was measured with the Tecan Infinite series M200 microplate reader.

### 4.8.1 Western blot

Western blot analysis was performed on whole-cell lysates. Equivalent amounts of proteins (10-30 µg per lane) were diluted with 4x Laemmli Sample Buffer (containing 77.8 mM Tris-HCl, pH 6.8, 4.4% w/v glycerol, 0.02% bromophenol blue) and 0.1 M DTT. After denaturation at 95°C for 5 minutes, the samples were loaded and separated onto 8-12.5% SDS-polyacrylamide gels at 90-120V. Precision Plus dual color Standard was used as protein standard. After separation on the gel, the proteins were transferred to a nitrocellulose membrane at 0.35A during 60 minutes. Successful transfer was visualized by Ponceau S staining. The red Ponceau S staining was washed out through washing with PBS and the membrane was blocked in 5% nonfat dry milk or 5% BSA in PBS/ 0.2% Tween 20 at room temperature for 1 hr. After blocking, the membrane was incubated with the primary antibody over night at 4°C (antibody diluted in 5% BSA in PBS/ 0.2% Tween 20). To remove unbound primary antibodies, the membrane was washed 3x 10 minutes with 1% nonfat dry milk in PBS/ 0.2% Tween 20. Incubation with secondary antibodies was performed at room temperature for 1 hr (antibody dissolved in 1% nonfat dry milk in PBS/ 0.2% Tween 20). The membrane was washed 3x 10 minutes with PBS/ 0.2% Tween 20 before detection of the protein. For peroxidase-conjugated secondary antibodies, peroxidase activity was detected using ECL and the membranes were exposed to X-OMAT LS films or measured with the LI-COR C-DiGit Blot Scanner. For IRDye680 and 800 conjugated antibodies, the membranes were analyzed with the Odyssey LI-COR system.

For successive detection of additional proteins, the membrane was washed with PBS/ 0.2% Tween 20 after analysis of the first protein, incubated for 30 minutes with the stripping buffer 1 (0.1 M glycine in PBS, pH 2-3) and subsequently shortly washed in stripping buffer 2 (1 M NaCl in PBS). Subsequently the membranes were washed three times with PBS and the protein detection procedure restarted from blocking process.

## 4.9 MicroRNA and pri-microRNA expression analysis

For the analysis of miRNA expression levels, **HUVECs** were stimulated 24 hr by 10 nM E2 in starving media whereas **HCASMCs** were induced by PDGF-BB (20 ng/ml) for 24 hr in the presence or absence of E2 (10-100 nM) in starving media. The role of

estrogen receptors (ERs) on miRNA expression was studied using ER agonists PPT, DPN and G1 (100 nM). ER antagonists MPP (500 nM) and ICI (1  $\mu$ g) were added 1 hr prior to E2 or the ER-agonists. Controls of both cell types were treated with vehicle (maximal 0.1% DMSO). Additionally, expression of miR-221 and miR-193a was assessed at 24, 48 and 72 hr after transfection with Mimics and Antimirs, and their respective controls, to confirm overexpression by the Mimics and silencing by the Antimir.

For pri-miRNA expression analysis, HUVECs were stimulated 24 hr by 10 nM E2 in starving media whereas HCASMCs were induced by PDGF-BB (20 ng/ml) for 24 hr in the presence or absence of E2 (10-100 nM) in starving media.

#### **4.9.1 RNA extraction and quantification**

Total RNA, including small RNAs, was extracted from the treated cells using the Quick-RNA MiniPrep Kit (ZymoResearch) according to the manufacture's protocol. Briefly, the cells were lysed directly in the culture dish by removing the culture media, adding RNA lysis buffer and collecting the lysate. Samples homogenized in lysis buffer can be stored frozen and processed at a later time. All centrifugation steps were performed at 10'000 x g. Lysates were cleared by centrifugation for 1 min before the supernatant was transferred into a Spin-Away Filter in a collection tube and centrifuged again to remove gDNA. The flow-through was saved for RNA purification. The same volume of ethanol (95-100%) was added to the sample and the solution was mixed well. The mixture was transferred to a Zymo-Spin IIICG Column in a collection tube and centrifuged 30 seconds. The flow through was discharged. For the optional In-column DNase I treatment, the column was prewashed with 400  $\mu$ l RNA Wash Buffer, centrifuged 30 seconds and flow-through discarded, before 80  $\mu$ l DNase I reaction mix (5  $\mu$ l DNase I and 75  $\mu$ l DNA digestion buffer) was added directly to the column matrix and incubated for 15 minutes at room temperature. The following washing steps were followed by 30 seconds centrifugation and discharge of flow-through. The column was washed by 400  $\mu$ l RNA Prep Buffer, 700  $\mu$ l RNA Wash Buffer and finally by 400  $\mu$ l RNA Wash Buffer, followed by 2 minutes of centrifugation. The RNA was eluted from the column by 50  $\mu$ l DNase/ RNase-free water, through 30 seconds of centrifugation and placed immediately on ice. The purified RNA could be stored at -80°C or directly processes to the reverse transcription, as described below.



The concentration of the total RNA was quantified by the absorbance at 260 nm using a NanoDrop system or Infinite 200 NanoQuant (Tecan). The overall quality of the RNA preparation purified with the Quick-RNA MiniPrep Kit was assessed on electrophoresis on a 1% denaturing agarose gel. Further RNA purity was considered by the ratios  $A_{260}/A_{280} > 1.8$  and  $A_{260}/A_{230} > 1.8$ .

#### 4.9.2 MicroRNA RT-qPCR

RT-qPCR for miRNAs was performed using the TaqMan miRNA assays (Life Technologies) which provides miRNA-specific RT primers as well as primers and probes for amplification and detection of the miRNA. Briefly, single-stranded complementary DNA (cDNA) was synthesized from 10 ng total RNA in 15  $\mu$ l reaction volume with the TaqMan miRNA Reverse Transcription Kit. Each 15  $\mu$ l reaction contained 1 mM deoxynucleotide (dNTP) mix, 50 U MultiScribe Reverse Transcriptase, 1x Reverse Transcription Buffer, 0.3 U RNase Inhibitor and 1x miRNA-specific RT primers. The reaction was incubated at 16°C for 30 minutes followed by 30 minutes at 42°C and inactivation at 85°C for 5 minutes. Samples were chilled on ice and diluted by addition of 75  $\mu$ l DEPC treated water. cDNA was stored at -20°C.

Amplification and detection of the specific products were performed on a Bio-Rad CFX Real-Time PCR Detection System. PCR reaction included 5  $\mu$ l 2x TaqMan Fast Advanced Master mix, 0.5  $\mu$ l each 20x TaqMan miRNA Assay mix, 0.5  $\mu$ l DEPC treated water and 4  $\mu$ l cDNA. The PCR reaction plate was run as following: 2 minutes at 50°C and 20 seconds at 95°C, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

As an internal control, RNU48 and RNU49 were used for miRNA template normalization. The relative gene expression was determined using the  $2^{-\Delta\Delta C_t}$  Method.

#### 4.9.3 Pri-microRNA RT-qPCR

The TaqMan High-Capacity cDNA Reverse Transcription Kit (Life Technologies) was used to synthesize single-stranded cDNA from the extracted RNA. Briefly, 20  $\mu$ l reaction contained 1x Reverse Transcription Buffer, 1x RT Random Primers, 4 mM deoxynucleotide (dNTP) Mix, 50 U MultiScribe Reverse Transcriptase, nuclease-free water and 10  $\mu$ l of 20  $\mu$ g/ $\mu$ l RNA. The reaction was incubated at 25°C for 10 minutes

followed by 120 minutes at 37°C and inactivation at 85°C for 5 minutes. cDNA was stored at -20°C or placed on ice for immediate procession with qPCR.

Amplification and detection of the specific products were performed on a Bio-Rad CFX Real-Time PCR Detection System. PCR reaction included 5 µl 2x TaqMan Fast Advanced Master mix, 0.5 µl each 20x TaqMan pri-miRNA Assay mix, 2.5 µl DEPC treated water and 2 µl cDNA. The PCR reaction plate was run as following: 2 minutes at 50°C and 20 seconds at 95°C, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

As an internal control, *GAPDH* and *hPRT1* genes were used for pri-miRNA template normalization. The relative gene expression was determined using the  $2^{-\Delta\Delta C_t}$  Method.

#### **4.10 Statistical analysis**

All experiments were repeated a minimum of 3 times. Data are presented as mean  $\pm$  standard error. Unless stated differently, for statistical evaluation Student's t tests and ANOVA were used and statistical significance ( $p < 0.05$ ) was calculated using Fisher's Least Significant Difference test.

## 5 Results and Discussion

### 5.1 E2 regulates miRNAs in ECs

#### Objective

The beneficial effects of E2 on the endothelium are well known; however, the role of miRNAs in mediating these effects remains unclear. Hence, under this specific aim, we screened for E2-regulated miRNAs in ECs.

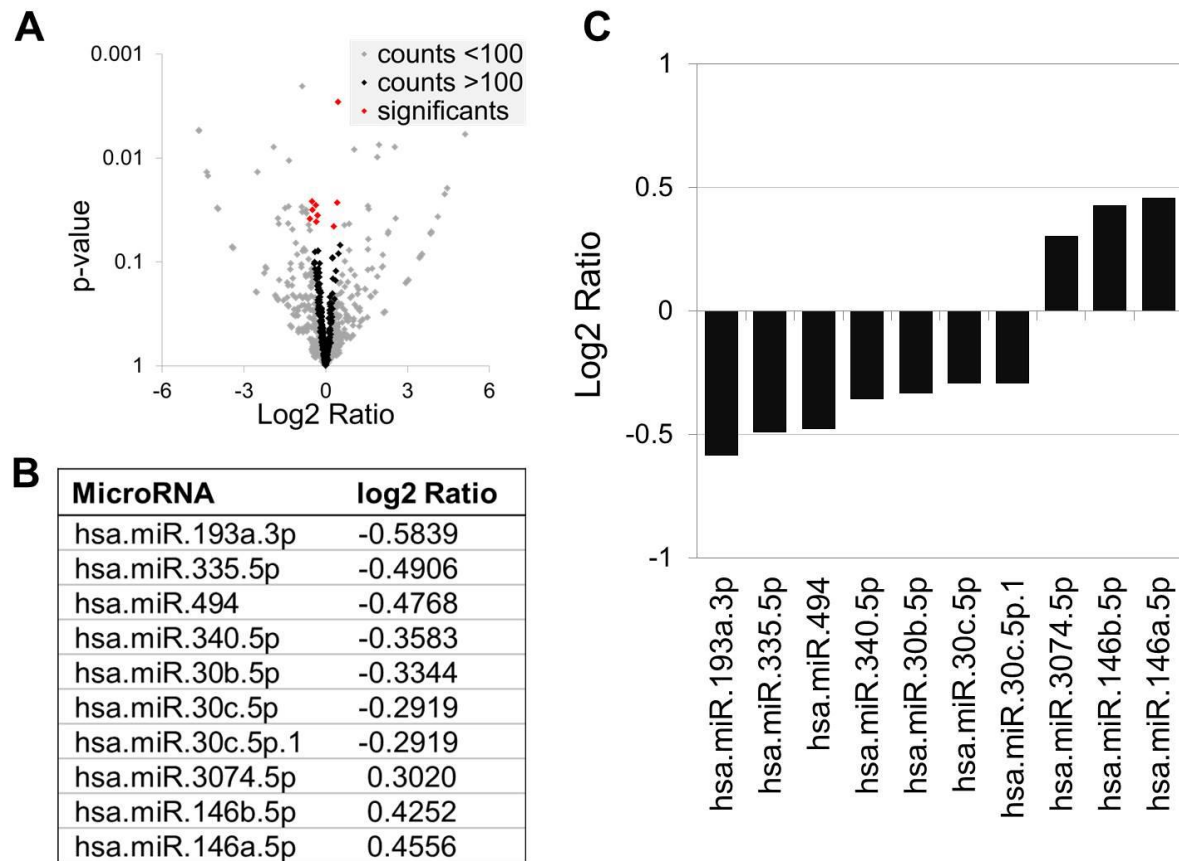
#### Introduction

The beneficial effects of E2 on endothelial growth and function are well established. For example, E2 promotes the recovery of injured or dysfunctional endothelium by stimulating EC proliferation, migration and capillary formation [130-132, 135, 136] and improving endothelial-dependent vascular relaxation [116]. Multiple intracellular signaling mechanism(s) have been identified in inducing the protective actions of E2 on ECs; however, the role of miRNAs in mediating these effects remains unclear.

Since miRNAs actively regulate ECs [208, 209] and are postulated to mediate estrogenic actions in cancer cells [88], we hypothesize that the protective actions of E2 on the vascular system are potentially mediated via regulation of miRNAs. To test our hypothesis, we screened for miRNAs that were regulated by E2 in ECs. To achieve this goal, we analyzed the data from a preliminary small RNA sequencing experiment in EPCs, searched the literature for interesting miRNAs and subsequently validated the selected miRNA candidates by RT-qPCR in HUVECs.

#### 5.1.1 E2 alters miRNA expression in EPCs

To investigate the role of miRNAs in mediating the protective actions of E2 in the vascular system, we first identified miRNAs that were differentially expressed in EPCs in response to E2. In this context, the cells were treated for 36 hr with or without 10 nM E2 and the RNA extracted. Subsequently a small RNA sequencing was performed using Illumina HiSeq2000 and the data analysed in collaboration with the Functional Genomic Center Zürich. Indeed, we found 10 miRNAs significantly regulated by E2 (**Figure 15**) - 7 down-regulated (miR-193a.3p, miR-335.5p, miR-494, miR-340.5p and miR-30b.5p, miR-30c.5p and miR-30c.5p.1) and 3 up-regulated (miR-146a.5p, miR-146b.5p and miR-3074.5p).

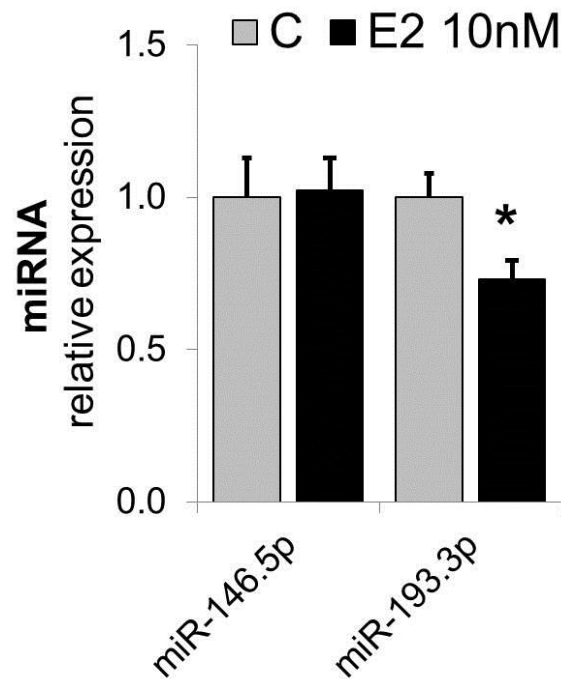


**Figure 15: Treatment with E2 alters miRNA expression in EPCs.** EPCs were seeded and grown to 60% confluency in complete media prior to 36 hr treatment with or without 10 nM E2 in triplicates. RNA was extracted and handled over to the Functional Genomic Center Zürich for RNA quality and quantity assessment and the following miRNA sequencing and data analysis. **Panel A:** Volcano plot of sequenced miRNAs. The miRNAs with single counts <100 (grey) were not analyzed for differential expression. Significantly regulated miRNAs are depicted in red. **Panel B and C:** Differentially expressed ( $p < 0.05$ ) miRNAs.

### 5.1.2 E2 down-regulates miR-193a in HUVECs

From the 10 differentially expressed miRNAs after treatment with E2 in EPCs, we picked out the two highly regulated, miR-193a.3p (log2 Ratio = -0.5839) and miR-146a.5p (log2 Ratio = 0.4556), to be validated by RT-qPCR in HUVECs.

We found that treatment with 10 nM E2 reduced the levels of miR-193a.3p by  $27 \pm 6\%$  ( $p < 0.05$ ), while it had no significant effect on miR-146a.5p (**Figure 16**), suggesting that E2 regulates only miR-193a.3p in HUVECs under our experimental conditions.

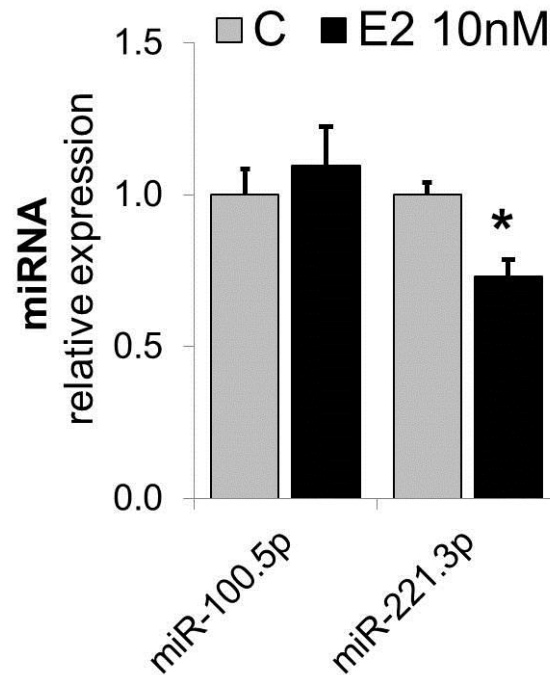


**Figure 16: Validation of E2-regulated miRNAs in HUVECs.** Cells were grown to 60% confluency in complete media prior to 24 hr treatment with or without 10 nM E2. Total RNA was extracted and the relative miRNA expression levels were determined by RT-qPCR using TaqMan miRNA assays. The results were normalized to U48 and U49.  $n=3$ ,  $*p<0.05$  compared to the respective control.

### 5.1.3 E2 down-regulates miR-221 expression in HUVECs

In addition to the miRNAs chosen from the EPC sequencing results, we tested the effect of E2 on miRNA-100 and miRNA-221, two miRNAs known to play a role in EC and vascular SMC function. It was demonstrated that miR-100 modulates proliferation, tube formation, and sprouting activity of ECs and migration of vascular SMCs [237]. Similarly, miR-221 decreases cell survival, migration and endothelial tube formation in HUVECs [215], whereas it induces growth of SMCs [230], which recalls the differential actions of E2 on EC and SMC growth.

In this study, we found that 10 nM E2 had no effect on miR-100 expression, while it inhibited the expression of miR-221 by  $26\pm5\%$  ( $p<0.05$ ; **Figure 17**), suggesting that in HUVECs miR-221, but not miR-100, is an estrogen responsive miRNA.



**Figure 17: E2 effect on miRNAs involved in EC regulation.** The miRNAs were chosen based on a literature search. HUVECs were grown to 60% confluency in complete media prior to 24 hr treatment with or without 10 nM E2. Total RNA was extracted and relative miRNA expression levels were determined by RT-qPCR using TaqMan miRNA assays. The results were normalized to U48 and U49. n=3, \*p<0.05 compared to the respective control.

#### 5.1.4 Discussion

The protective effects of estrogens in the cardiovascular system are well established and include the promotion of endothelial repair and regeneration; however, the mechanisms involved remain unclear. The role of miRNAs in the vasculature has been extensively studied [193-195]. Recently, estrogens have been associated with alterations in miRNA expression [83, 84] and several studies have shown that E2-regulated miRNAs mediate the actions of E2 in different tissues, including the vascular system [90-93]. Hence, to assess the role of miRNAs in mediating the protective effects of E2 in ECs, we screened for miRNAs regulated by E2 in EPCs by performing a small RNA sequencing experiment, plus through literature research, and validated the differential expression in HUVECs, our model to investigate EC functions.

Analysing the data from the small RNA sequencing, we identified 10 miRNAs significantly regulated by E2 in EPCs: 7 down-regulated (miR-193a.3p, miR-335.5p, miR-494, miR-340.5p and miR-30b.5p, miR-30c.5p and miR-30c.5p.1) and 3 up-

regulated (miR-3074.5p, miR-146b.5p and miR-146a.5p). Of the 10 identified miRNA, we decided to validate only the two most strongly regulated miRNAs by RT-qPCR in HUVECs: miR-193a.3p and miR-146a.5p.

Although no study has elucidated the role of miR-193a in vascular cells until now, few studies revealed that miR-193a modulates cancer cell growth, i.e. miR-193a regulates proliferation and apoptosis in epithelial ovarian cancer cells [238]. In addition, numerous studies have reported that miR-193a suppresses tumor development in different types of cancers [239-245]. Moreover, miR-193a overexpression inhibited tumor cell migration and invasion capabilities [246, 247]. In contrast, miR-146a has been shown to regulate vascular proliferation and angiogenesis. Overexpression of miR-146a enhanced angiogenic activity in HUVECs through the promotion of the pro-angiogenic factor FGF [248] and knock-down of miR-146a significantly reduced LPS-induced EC migration and angiogenesis [249].

Based on the above findings, both miRNAs are interesting targets, since it is conceivable that the stimulatory effects of E2 on EC growth and motility are potentially mediated via down-regulation of the anti-proliferative miR-193a and/ or induction of the pro-angiogenic miR-146a. However, validation of the two miRNAs in HUVECs confirmed only down-regulation of miR-193a by E2, while miR-146a was not altered. The controversial finding about miR-146a might be due to false positive results in the small RNA sequencing, or more likely caused by the phenotypic differences of cells i.e. EPCs and ECs.

In addition to the miRNAs chosen from the EPC sequencing results, we tested the effect of E2 on miRNA-100 and miRNA-221 expression in HUVECs, two miRNAs described to negatively influence EC function [215, 237]. We discovered that E2 altered miR-221 expression, while miR-100 was unaffected, suggesting that only miR-221 might mediate the protective actions of E2 in ECs. Since miR-221 adversely affects EC proliferation, migration and tube formation, it is very well possible that the stimulatory effects of E2 on EC function are mediated via the down-regulation of this miRNA.

The alterations in miRNA expression are generally rather small, both in the sequencing and in the RT-qPCR results. However, even relatively small changes in miRNA expression may be biologically significant, as the capability of miRNAs to

regulate multiple targets within the same pathway amplifies their biological effect [250, 251].

Taken together, we identified two E2-regulated miRNAs miR-193a and miR-221 in HUVECs. Moreover, we postulate that the beneficial effects of E2 on the endothelium and consequently on the vascular system are in part mediated via down-regulation of these miRNAs and abrogation of their potentially disruptive impact in ECs.



## 5.2 The role of miR-193a and miR-221 in ECs

### Objective

We identified two miRNAs that were regulated by E2 in HUVECs: miR-193a and miR-221. Hence, under this specific aim we investigated the effects of miR-193a and miR-221 on EC function and their role in mediating the beneficial effects of E2.

### Introduction

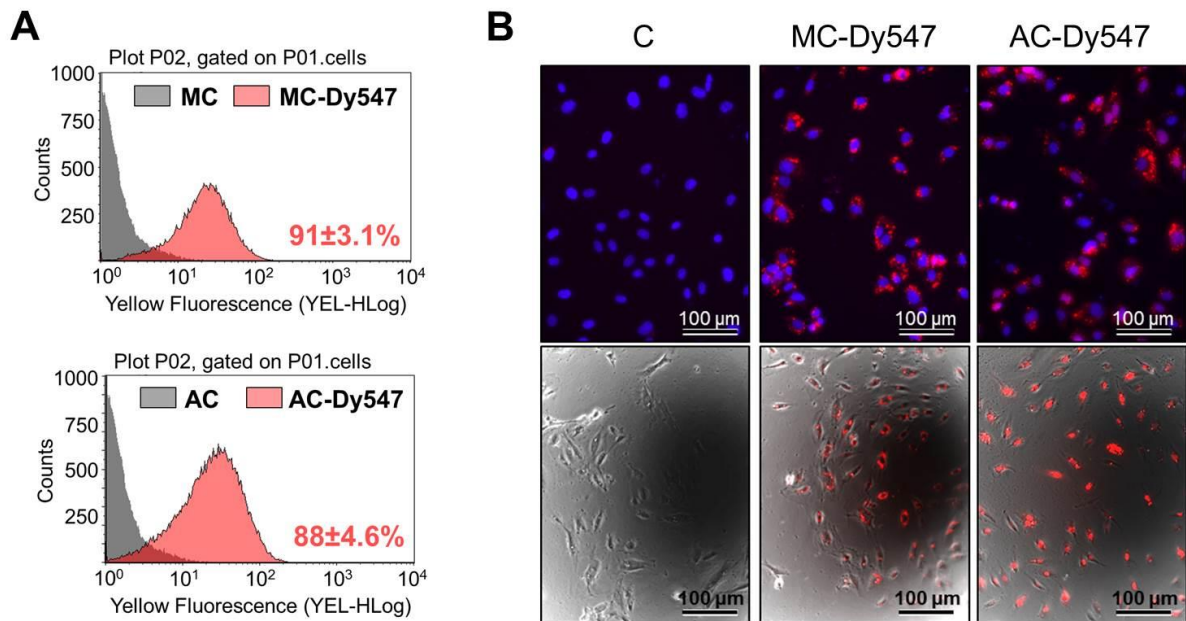
The function of miR-221 in the vascular endothelium has been elucidated by several studies. It has been shown to inhibit pro-angiogenic activation, proliferation and migration of ECs. Expression of miR-221 is increased at the onset of atherosclerosis and is possibly involved in maladaptive vascular remodeling as it increases EC dysfunction and apoptosis [215, 221]. Compared to miR-221, the impact of miR-193a on the vascular system is not known. However, it has been shown to be associated with the inhibition of proliferation and motility of different cancer cells [238-247].

Based on the above findings, we postulate that in ECs miR-193a may have similar physiological effects as miR-221. Moreover, their actions would be opposite to the actions of E2. Furthermore, we hypothesize that the stimulatory effects of E2 on ECs are, at least in part, due to the down-regulation of miR-193a and miR-221. To study this, we first confirmed the role of miR-221 and examined the effects of miR-193a on HUVEC vasculogenesis, migration and proliferation using synthetic Mimics and Antimirs to specifically modulate the expression of miRNAs. Second, we compared the effects of miRNA inhibition with the effects of E2. Finally we investigated the effects of miR-193a and miR-221 overexpression after treatment of E2.

### 5.2.1 Mimics and Antimirs dysregulate the expression of miRNAs

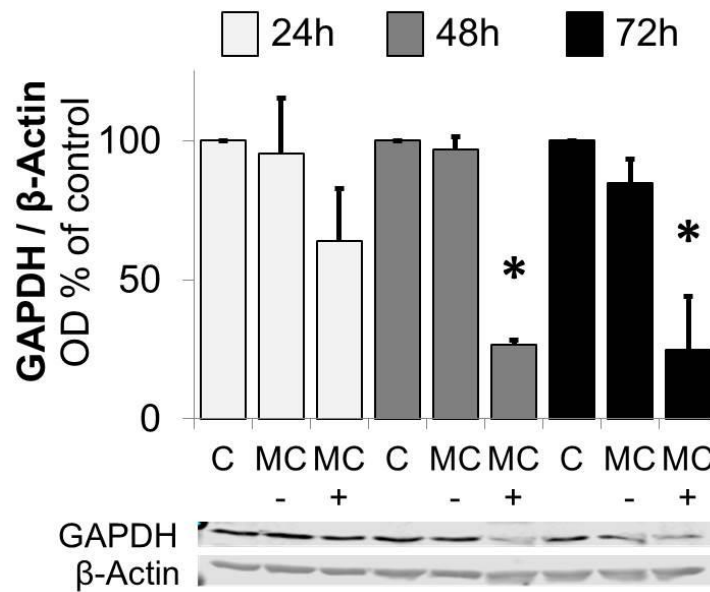
To determine the role of miR-193a and miR-221 in HUVECs, the cellular levels of these miRNAs were increased using miRNA Mimics (small double-stranded RNA molecule with the same sequence as the respective miRNA, thus able to “mimic” native miRNAs). Inhibition of both miRNAs was achieved using the respective miRNA Antimirs (single stranded RNA molecules designed to specifically bind endogenous miRNA molecules and prevent their activity). Both Mimics and Antimirs can be introduced into cells using transfection and enable detailed study of miRNA biological effects.

Transfection success was assessed using Dy547-labelled controls and quantified using flow cytometry. Transfection efficiency was  $91\pm3.1\%$  for the Dy547-labelled Control Mimic and  $88\pm4.6\%$  for the Dy547-labelled Control Antimir in HUVECs (**Figure 18A**). Representative images were taken with a fluorescence microscope to visualize the transfection efficiency (**Figure 18B**).



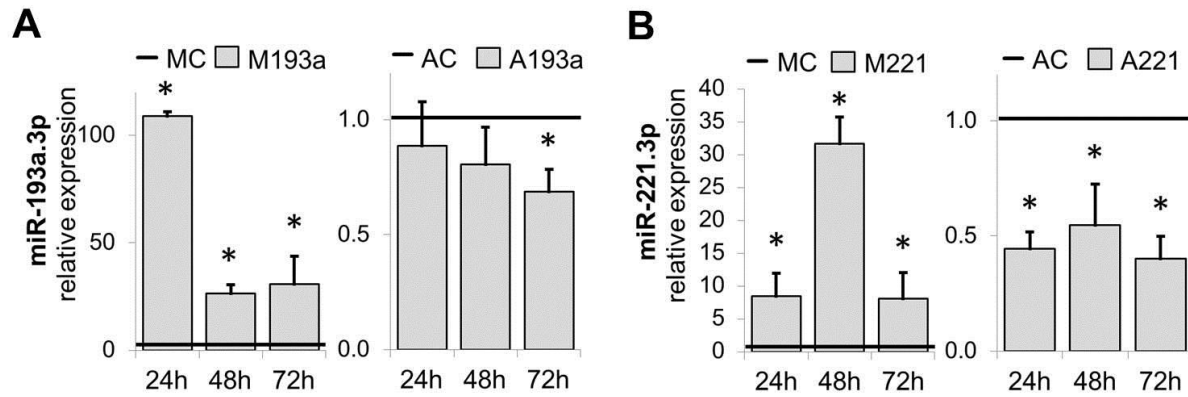
**Figure 18: Transfection efficiency of miRNA Mimics and Antimirs.** Cells were transfected with 25 nM of Dy547-labelled Control Mimic (MC-Dy547) or 25 nM of Dy547-labelled Control Antimir (AC-Dy547) using Lipofectamine 2000 for 6 hr in the absence of antibiotics and serum. **Panel A:** The percentage of Dy547-positive cells was measured using flow cytometry. **Panel B:** Representative images were taken using bright field and fluorescence microscopy (Red = Dy547-labeled Mimic or Antimir; blue = Hoechst33342 stain). n=3.

We further confirmed optimal transfection conditions by means of a Positive Control Mimic specifically targeting GAPDH. Protein expression of GAPDH was reduced by ~40% (n.s.) 24 hr post-transfection and by ~75% ( $p<0.05$ ) at 48 hr and 72 hr after transfection with the positive Control Mimic (**Figure 19**).



**Figure 19: Confirmation of optimal Mimic transfection conditions.** HUVECs transfected with 25 nM GAPDH-targeting Positive Control Mimic (MC+), Negative Control (MC-) or mock transfected (C) were kept in growing media for 24 hr, 48 hr and 72 hr prior to cell lysis and analysis of GAPDH protein expression using Western blot. β-Actin was used as a loading control. n=3, \*p<0.05 compared to MC-.

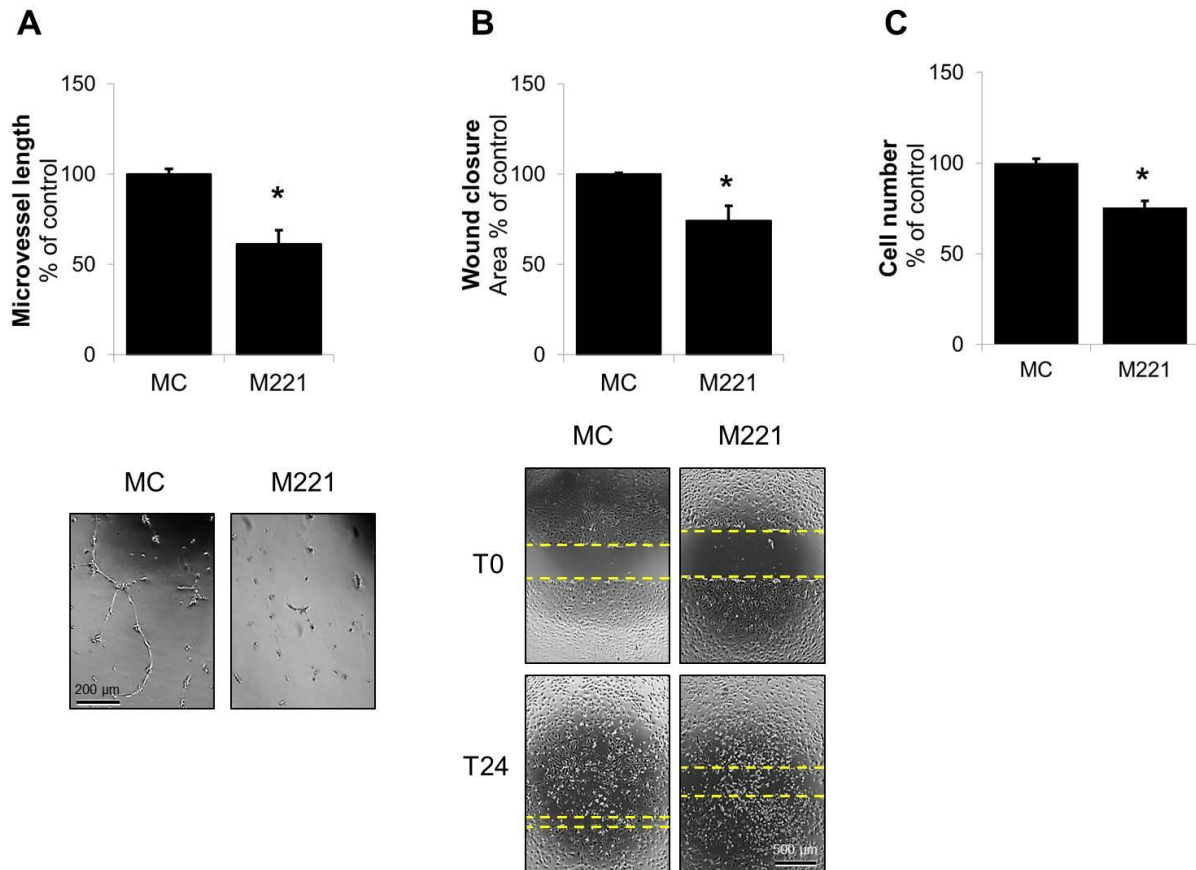
In order to confirm the alterations in miRNA levels by the Mimics and Antimirs, we isolated RNA from transfected cells and determined miR-193a as well as miR-221 levels by RT-qPCR (**Figure 20**). Both miR-193a and miR-221 Mimics (M193a and M221, respectively) substantially increased the levels of the respective miRNA after 24 hr, 48 hr and 72 hr. Transfection with the Antimir of miR-193a resulted in a time dependent reduction in miR-193a levels, which was significant after 72 hr ( $32 \pm 9.78\%$  reduction,  $p < 0.05$ ). In case of miR-221, its levels were inhibited by  $\sim 50\%$  ( $p < 0.05$ ) at each tested time point by its Antimir.



**Figure 20: Transfection with Mimics and Antimirs changes the level of the respective miRNA.** HUVECs were transfected either with 25 nM miR-193a Mimic (M193a) or miR-193a Antimir (A193a; **Panel A**) or 25 nM miR-221 Mimic (M221) or miR-221 Antimir (A221; **Panel B**) and the respective negative controls (MC, Mimic Control; AC, Antimir Control). The cells were kept in growing media 24 hr, 48 hr and 72 hr post-transfection before RNA was extracted and the miRNA levels determined by RT-qPCR using TaqMan miRNA assays. The results were normalized to U48 and U49. n=3, \*p<0.05 compared to the respective control.

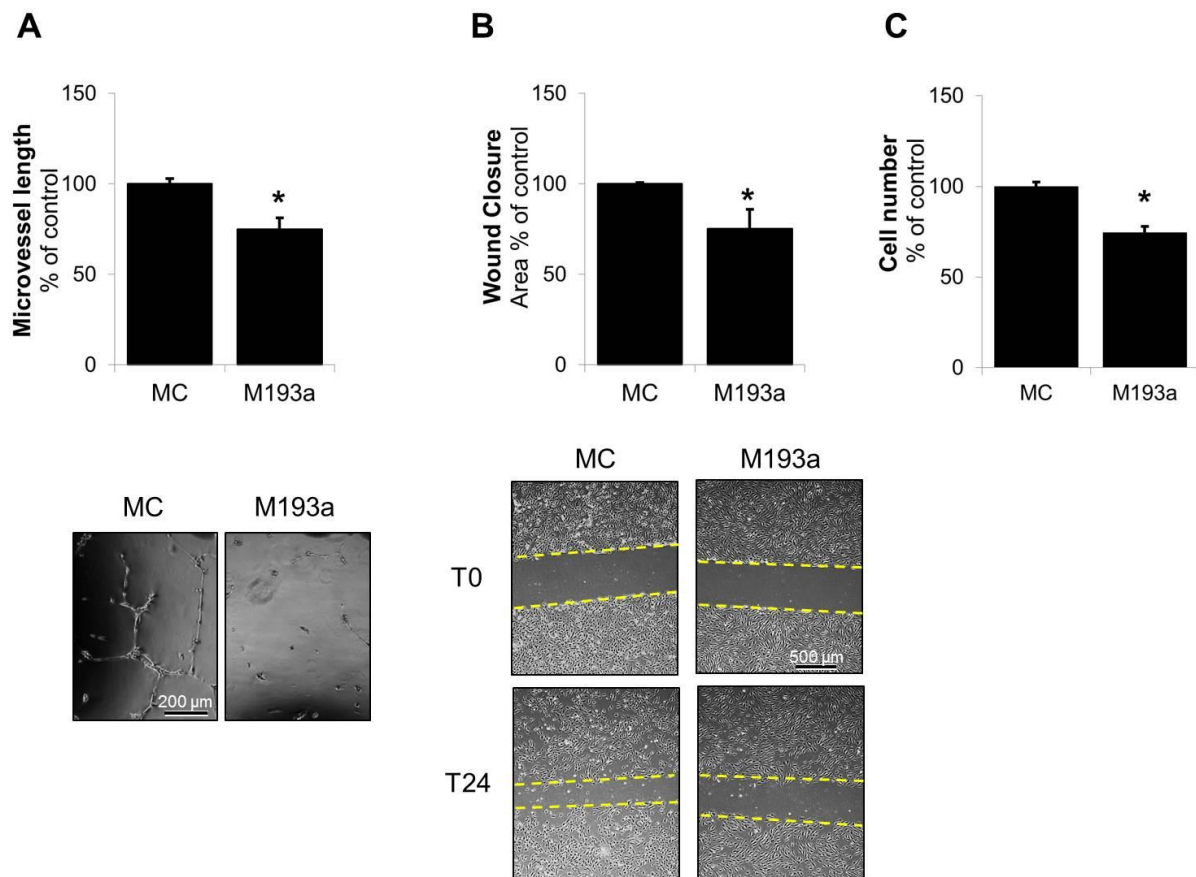
### 5.2.2 miR-193a and miR-221 inhibit HUVEC function

We first confirmed the inhibitory role of miR-221 on EC function. Transfection of HUVECs with miR-221 Mimic significantly reduced capillary formation in a matrigel-based assay by  $39\pm 8\%$  (**Figure 21A**). Moreover, increased levels of miR-221 inhibited cell migration by  $25\pm 8\%$  ( $p<0.05$ ) as assessed by an *in vitro* wound closure assay (**Figure 21B**). Finally we found that HUVEC proliferation was reduced (from  $100\pm 2\%$  to  $75\pm 4\%$ ,  $p<0.05$ ; **Figure 21C**).



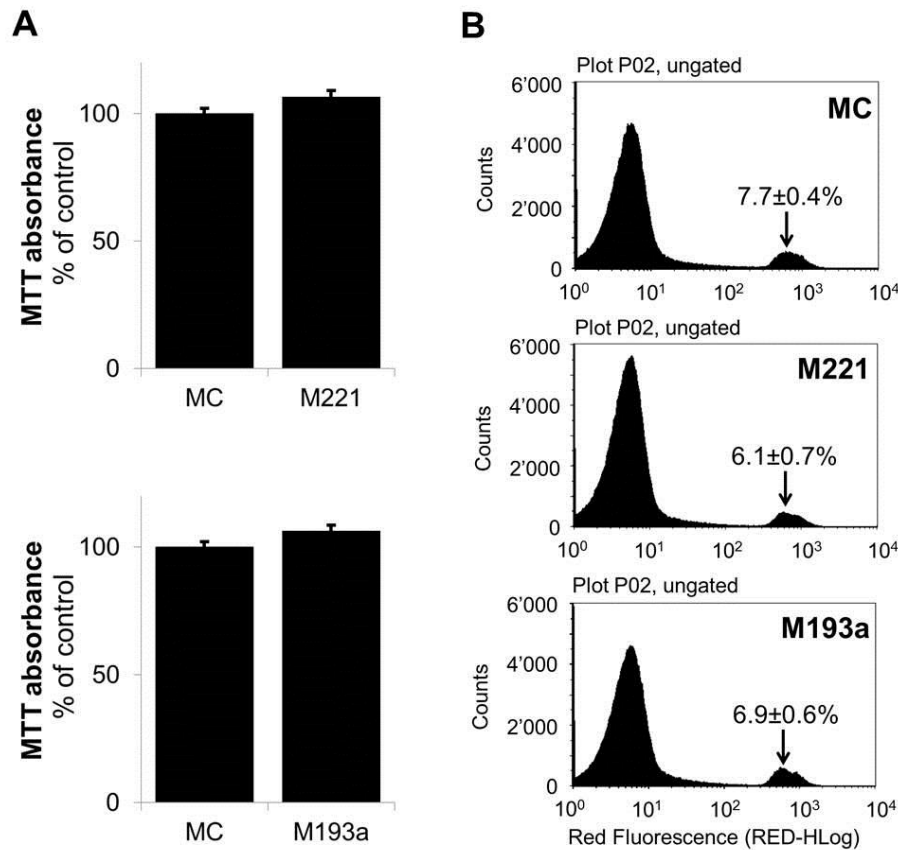
**Figure 21: miR-221 overexpression inhibits EC function.** Prior to use in various assays, HUVECs were transfected with miR-221 Mimic (M221) or Control Mimic (MC). **Panel A:** Microvessel formation was investigated using matrigel-based assay. HUVECs were allowed to form tube-like structures for 16-18 hr, before tube length was measured. Photomicrographs depict representative images under each treatment condition. **Panel B:** Migration was investigated by a wound closure assay. Images represent scratch wounds at time 0 (T0) and after 24 hr (T24). **Panel C:** Proliferation was assessed by counting of the cells 3 days after transfection.  $n=3$ ,  $*p<0.05$  compared to the respective control.

In parallel experiments, we investigated the role of miR-193a in ECs. Analogous to miR-221, miR-193a Mimic significantly inhibited the formation of capillaries in the matrigel-based assay by  $25\pm6\%$  (**Figure 22A**). Further M193a decreased HUVEC migration, as wound closure was reduced by  $25\pm11\%$  ( $p<0.05$ ; **Figure 22B**), and reduced cell proliferation (from  $100\pm2\%$  to  $74\pm4\%$ ,  $p<0.05$ ; **Figure 22C**).



**Figure 22: miR-193a overexpression inhibits EC function.** Prior to use in various assays, HUVECs were transfected with miR-193a Mimic (M193a) or Control Mimic (MC). **Panel A:** Microvessel formation was investigated using matrigel-based assay. HUVECs were allowed to form tube-like structures for 16-18 hr and tube length was measured. Photomicrographs depict representative images under each treatment condition. **Panel B:** Migration was investigated by a wound closure assay. Images represent scratch wounds at time 0 (T0) and after 24 hr (T24). **Panel C:** Proliferation was assessed by counting of the cells 3 days after transfection.  $n=3$ ,  $*p<0.05$  compared to the respective control.

Since inhibition of capillary formation, migration and proliferation could be a result of impaired cell viability, we verified whether overexpression of miR-221 or miR-193a increases cell death. Interestingly, neither of the two miRNAs affected cell viability, as assessed by MTT test and propidium iodide (PI) staining followed by flow cytometry analysis (**Figure 23A and 23B**). M221 showed only  $6\pm 2\%$  (n.s.) and M193a  $5\pm 2\%$  (n.s.) difference in MTT absorbance compared to the transfection of the cells with the Control Mimic. These results were confirmed by PI staining: MC displayed  $7.7\pm 0.4\%$ , M221 had  $6.1\pm 0.7\%$  and M193a showed  $6.9\pm 0.6\%$  PI positive cells.

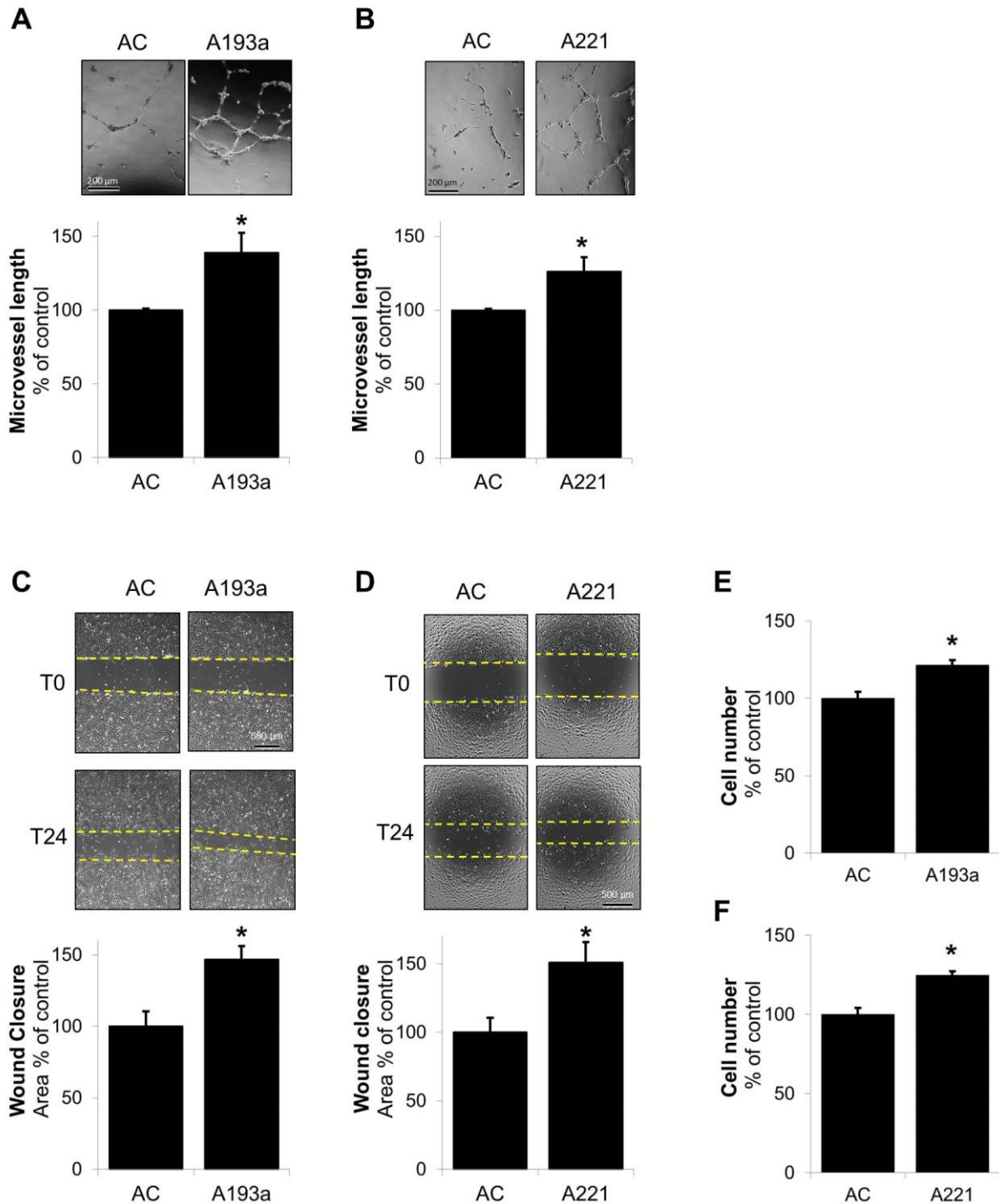


**Figure 23: miR-221 and miR-193a overexpression does not affect EC survival.** HUVECs were transfected with miR-221 Mimic (M221), miR-193a Mimic (M193a) or the Control Mimic (MC) and cultured in growing media for 24 hr after transfection. **Panel A:** For MTT assay the cells were incubated 1 hr with 0.5 mg/ml MTT prior to absorbance measurement. **Panel B:** For PI staining the cells were trypsinized, incubated for 5 minutes with 0.2 µg/ml PI and analyzed for viability with a flow cytometer. The percentage of dead cells is indicated. n=3.

### 5.2.3 miR-193a and miR-221 Antimirs mimic the effects of E2

As expected, down-regulation of the miRNAs by the Antimirs showed opposite outcomes in respect to the previously shown up-regulation by the Mimics. Capillary formation was significantly increased by A193a and A221, from 100±1% to 139±13% and 126±9%, respectively (**Figure 24A and 24B**). Moreover, neutralization of miR-193a and miR-221 with the respective Antimirs stimulated wound closure/ cell migration by 47±9% and 51±15%, respectively ( $p < 0.05$ ; **Figure 24C and 24D**). Furthermore, the Antimirs significantly induced cell proliferation by 21±3% (A193a) and 25±2% (A221; **Figure 24E and 24F**).

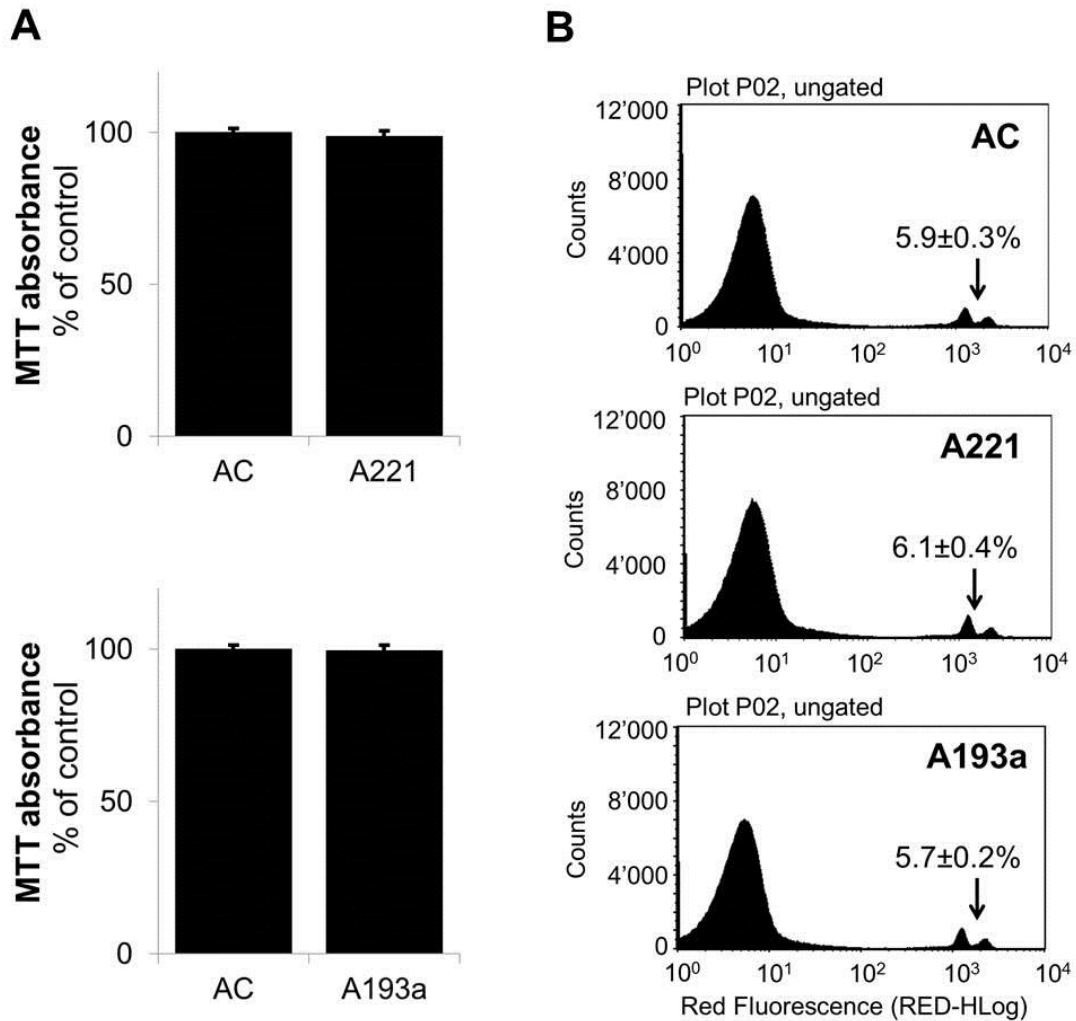




**Figure 24: miR-193a and miR-221 down-regulation stimulates vasculogenesis, migration and proliferation in HUVECs.** Prior to being employed for various assays, HUVECs were transfected with miR-193a Antimir (A193a), miR-221 Antimir (A221) or the Control Antimir (AC). **Panel A and B:** Tube formation was investigated using matrigel-based assay. HUVECs were allowed to form tube-like structures for 16-18 hr and tube length was measured. Photomicrographs depict representative images for each condition. **Panel C and D:** Migration was investigated by a wound closure assay. Images represent scratch wounds at time 0 (T0) and after 24 hr (T24). **Panel E and F:** Proliferation was assessed by counting of the cells 3 days after transfection. n=3, \*p<0.05 compared to the respective control.



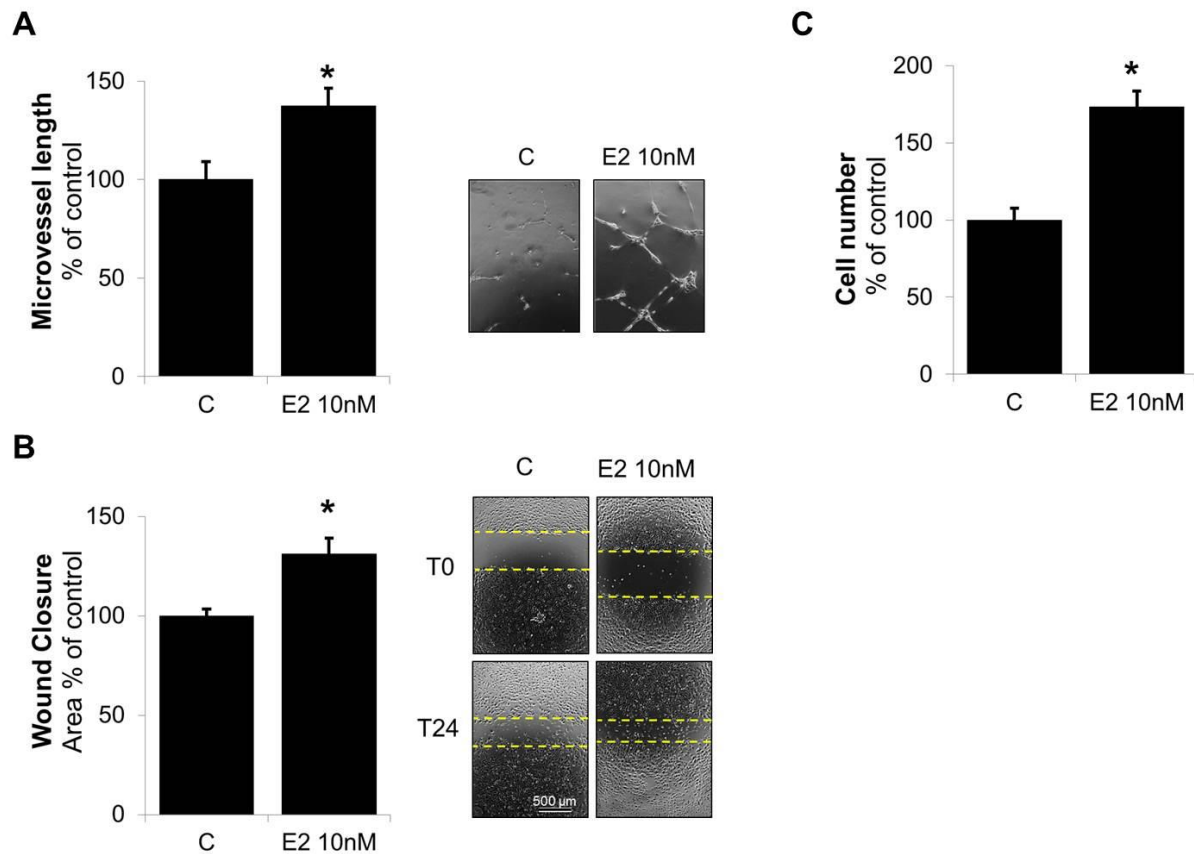
As for the Mimics, we assessed the role of the Antimirs on cell viability using MTT assay and PI staining followed by flow cytometry analysis (**Figure 25A and 25B**). We found that silencing of miR-193a and miR-221 did not influence MTT absorbance or the profile of the PI-stained cells. The percent of non-viable cells in AC, A221 and A139a was  $5.9\pm0.2\%$ ,  $6.1\pm3\%$  and  $5.7\pm0.2\%$ , respectively.



**Figure 25: miR-221 and miR-193a down-regulation does not affect EC survival.** HUVECs were transfected with the Antimir for miR-221 (A221), miR-193a (A193a) or Control Antimir (AC) and cultured in growing media for 24 hr after transfection. **Panel A:** For MTT assay the cells were incubated 1 hr with 0.5 mg/ml MTT prior to absorbance measurement. **Panel B:** For PI staining the cells were trypsinized, incubated for 5 minutes with 0.2  $\mu$ g/ml PI and analyzed for viability in a flow cytometer. The percentage of dead cells is indicated.  $n=3$ .

Down-regulation of miR-221 and miR-193a in HUVECs had similar outcomes as treatment with E2, which is known to positively influence the endothelium through induction of EC recovery. Indeed, E2 stimulated capillary formation (from  $100\pm9\%$  to

137±9%,  $p<0.05$ ) and induced wound closure (from 100±4 to 131±8%,  $p<0.05$ ; **Figure 26A and 26B**). Further, E2 significantly induced cell proliferation by 73±10% (**Figure 26C**).

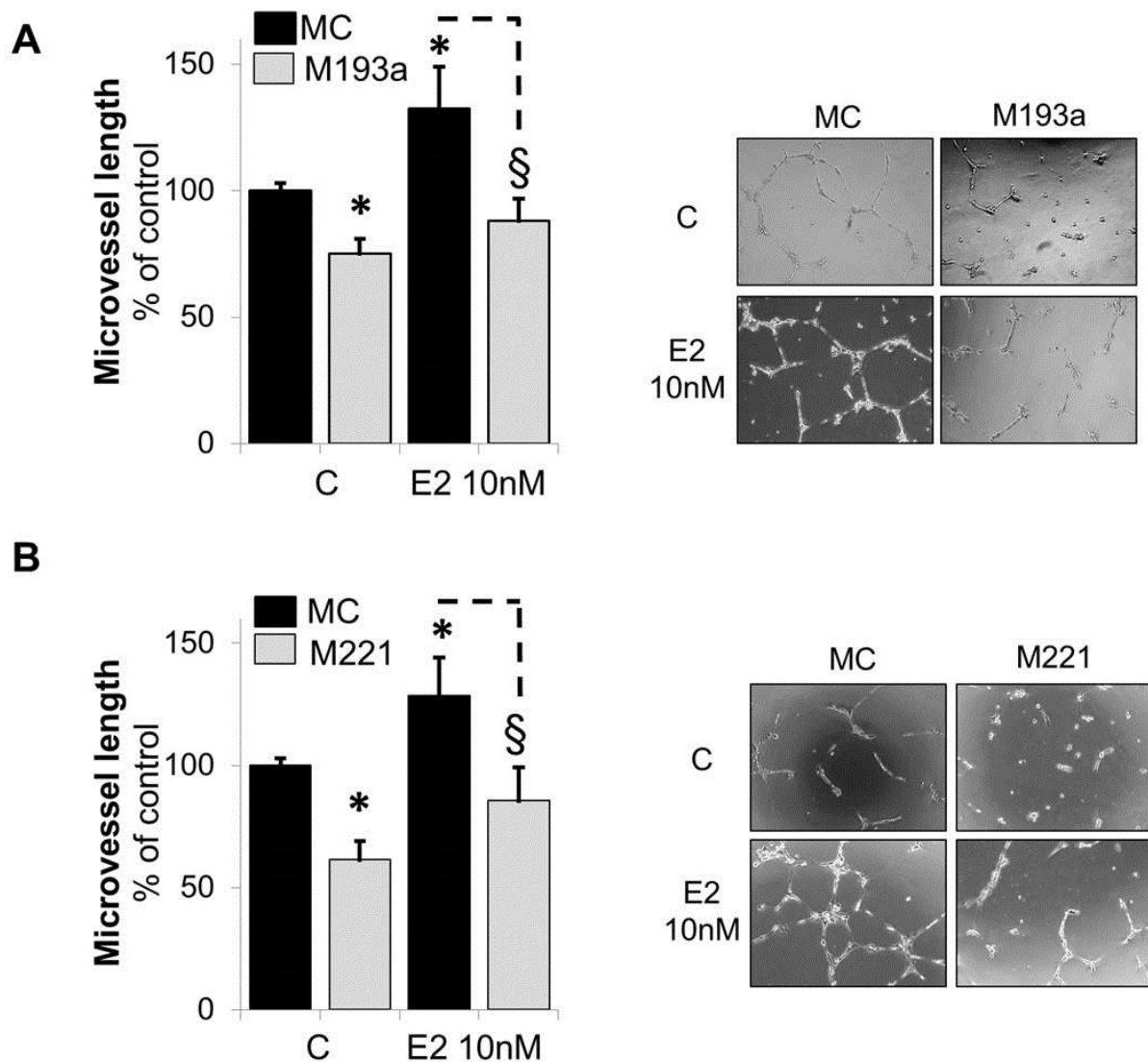


**Figure 26: E2 induces EC recovery.** HUVECs were treated with 10 nM E2 or DMSO vehicle as a control (C) before being used in different assays. **Panel A:** Microvessel formation was investigated using matrigel-based assay. Cells were allowed to form tube-like structures for 16-18 hr. Tube length was measured microscopically. Photomicrographs depict representative images for each condition. **Panel B:** Migration was investigated by a wound closure assay. Images represent scratch wounds at time 0 (T0) and after 24 hr (T24). **Panel C:** Proliferation was assessed by counting of the cells on day 3 after treatment.  $n=3$ ,  $*p<0.05$  compared to the respective control.

#### 5.2.4 miR-193a and miR-221 abrogate the protective actions of E2

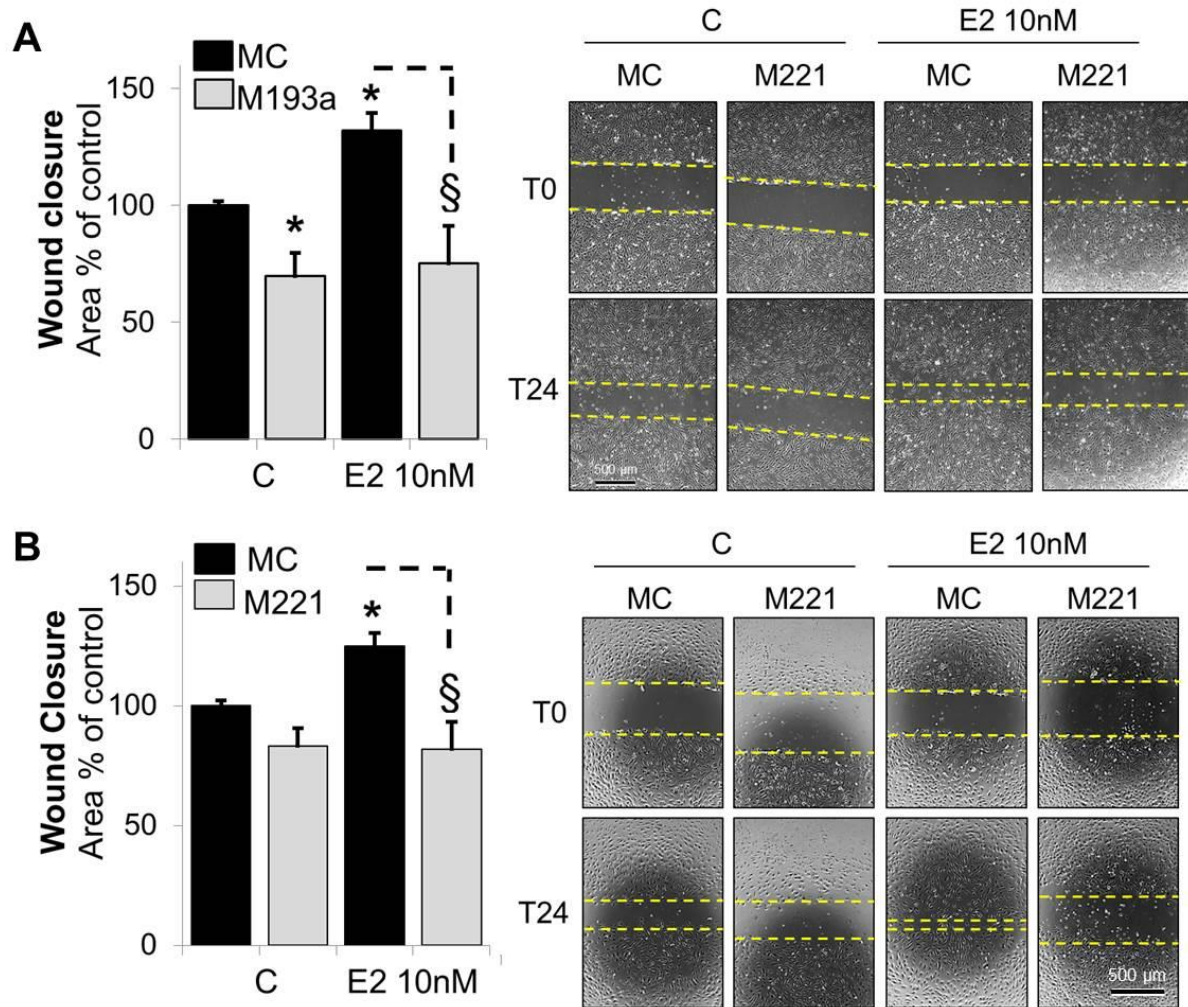
Based on our observations that E2 inhibits miR-193a and miR-221 expression and that down-regulation of both miRNAs mimics the effects of E2 on HUVECs, we hypothesized that modulation of miR-193a and miR-221 by E2 participates in mediating the protective effect of E2 in ECs. In order to verify this hypothesis, the effect of E2 was assessed and compared in HUVECs overexpressing miR-193a or miR-221. Indeed, the stimulatory effect of E2 on capillary formation was significantly

inhibited in HUVECs transfected with M193a (from  $133\pm 17\%$  to  $88\pm 9\%$ ); **Figure 27A**) or M221 (from  $137\pm 9\%$  to  $85\pm 13\%$ ; **Figure 27B**).



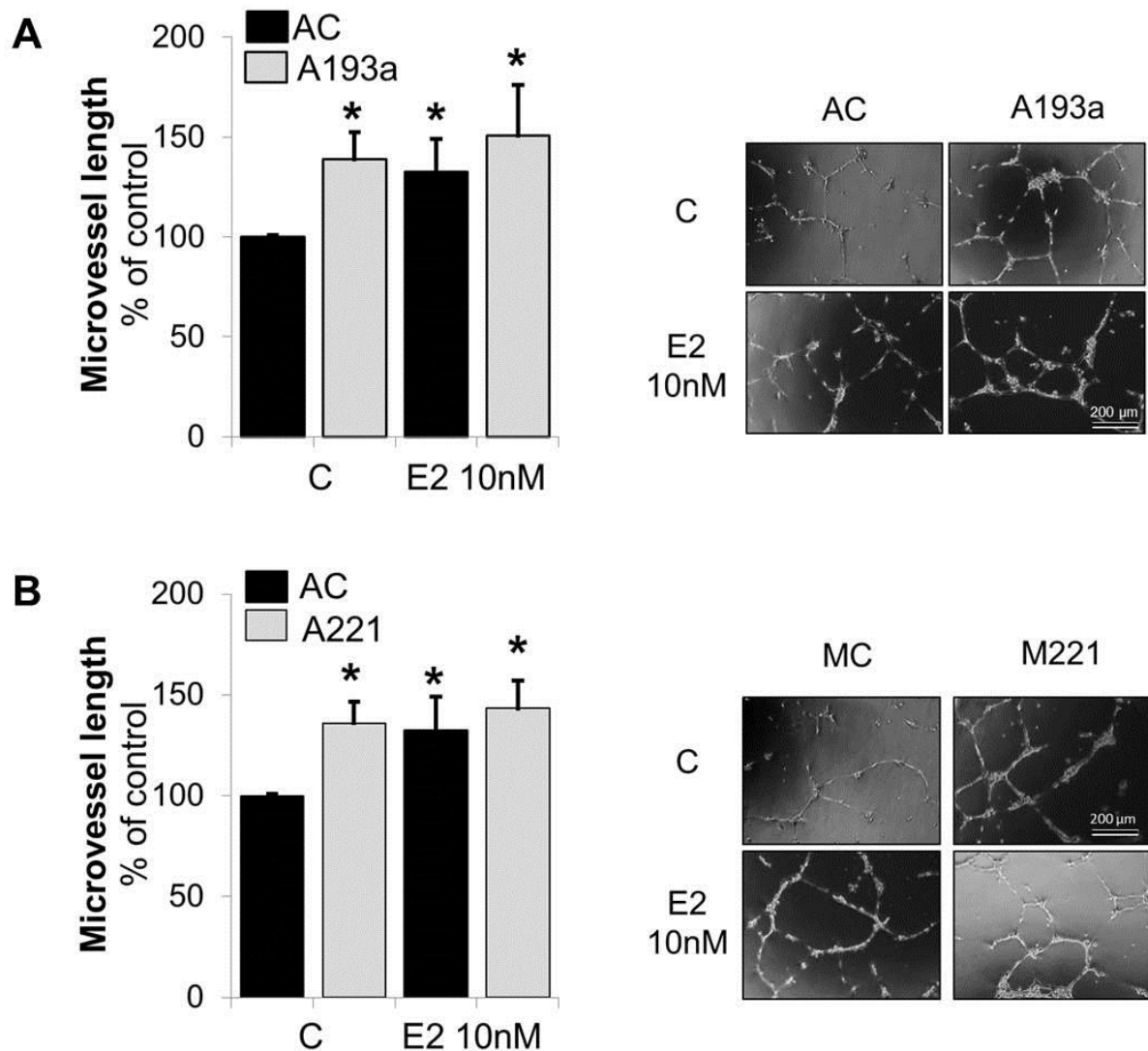
**Figure 27: miR-193a and miR-221 overexpression reduces the angiogenic actions of E2 in HUVECs.** Cells were transfected with Control Mimic (MC), miR-193a Mimic (M193a; **Panel A**) or miR-221 Mimic (M221; **Panel B**) prior to analysis of microvessel formation. Cells were incubated for 30 minutes with and without 10 nM E2 in serum free media before plating on matrigel. Tube length was measured after 16-18 hr. Figures depict representative photomicrographs.  $n=3$ ,  $*p<0.05$  compared to the respective control,  $\S p<0.05$  as indicated.

Likewise, the stimulatory effect of E2 on migration was significantly inhibited by up-regulation of both miR-193a (from  $132\pm 7\%$  to  $75\pm 16\%$ ; **Figure 28A**) and miR-221 (from  $125\pm 5\%$  to  $82\pm 12\%$ ; **Figure 28B**).



**Figure 28: miR-193a and miR-221 overexpression abrogates the mitogenic actions of E2 in HUVECs.** Cells were transfected with Control Mimic (MC), miR-193a Mimic (M193a; **Panel A**) or miR-221 Mimic (M221; **Panel B**) before assessing migration by wound closure assay. HUVECs were scratched before treatment with 10 nM E2 for 24 hr. Images represent scratch at time 0 (T0) and 24 hr later (T24).  $n=3$ , \* $p<0.05$  compared to the respective control, § $p<0.05$  as indicated.

Moreover, in support of our hypothesis that down-regulation of miR-193a and miR-221 by E2 mediates the protective effect of E2 in HUVECs, we found that the angiogenic actions of E2 were not enhanced after down-regulation of the miRNAs with Antimirs. E2 induced tube formation from  $100 \pm 2\%$  to  $133 \pm 15\%$  ( $p<0.05$ ) in cells transfected with Control Antimir, whereas in combination with A193a (**Figure 29A**) or A221 (**Figure 29B**) tube formation increased non-significantly from  $133 \pm 15\%$  to  $151 \pm 25\%$  (n.s.) and to  $143 \pm 14\%$  (n.s.), respectively.



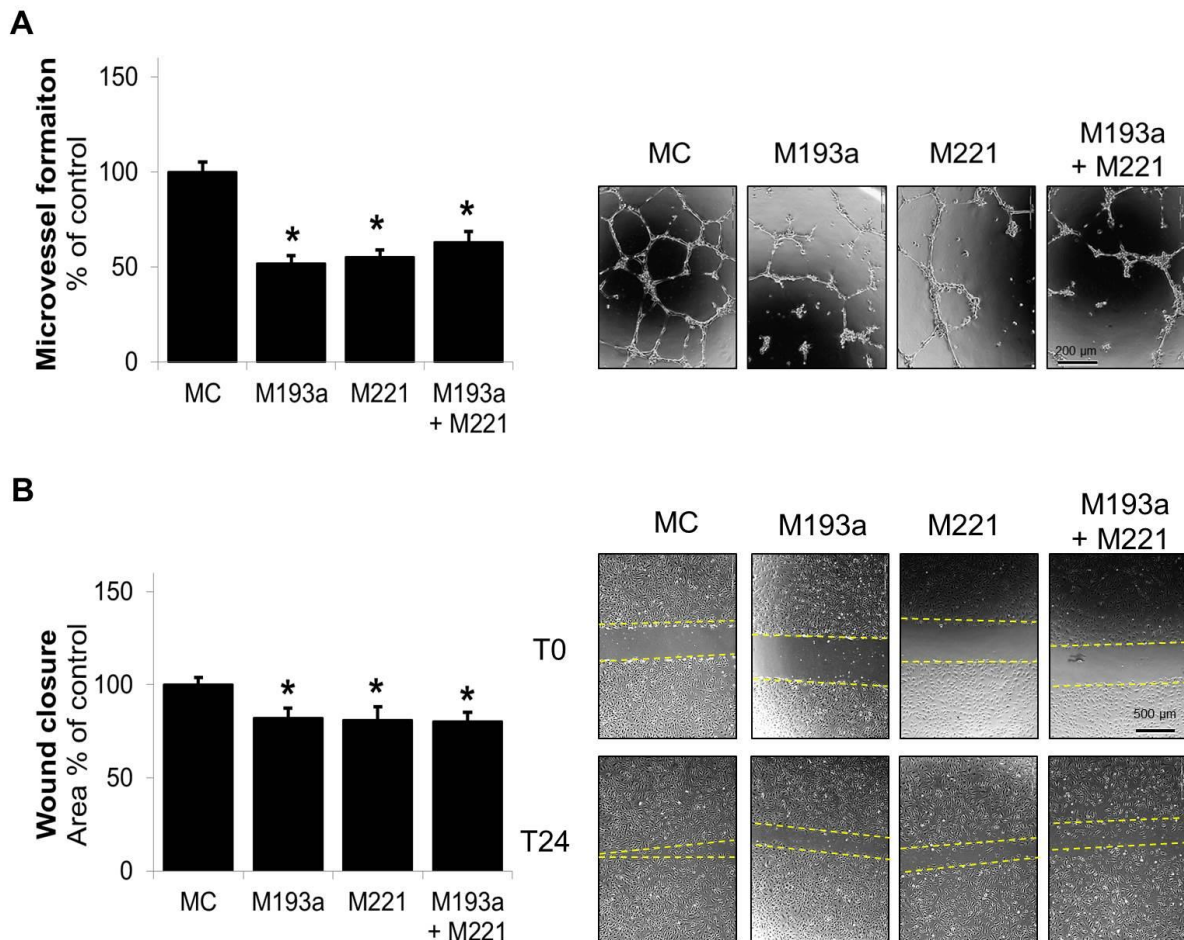
**Figure 29: miR-193a and miR-221 down-regulation does not enhance the angiogenic actions of E2 in HUVECs.** Cells were transfected with Control Antimir (AC), miR-193a Antimir (A193a; **Panel A**) or miR-221 Antimir (A221; **Panel B**) prior to analysis of microvessel formation. Cells were incubated for 30 minutes with and without 10 nM E2 in serum free media before plating on matrigel for 16-18 hr. Figures depict representative photomicrographs.  $n=3$ ,  $*p<0.05$  compared to the respective control.

### 5.2.5 miR-193a and miR-221 do not have additive effects

Since both miRNAs have a similar inhibitory role in HUVECs, we examined the effect of the joint overexpression of the two miRNAs on microvessel formation (**Figure 30A**) and wound closure (**Figure 30B**). We found that, similar to the individual Mimics, the combination of the Mimics significantly reduced both microvessel formation and wound closure by  $37\pm5\%$  or  $20\pm3\%$ , respectively. Importantly, there was no additive effect when compared to the individual Mimics M193a and M221, which inhibited



microvessel formation by  $48\pm4\%$  and  $45\pm4\%$ , respectively ( $p<0.05$ ), and decreased wound closure by  $18\pm5\%$  and  $19\pm7\%$  respectively ( $p<0.05$ ).



**Figure 30: Combined miR-193a and miR-221 overexpression has no additive effect.** Cells were transfected with either 25 nM Control Mimic (MC), miR-221 Mimic (M221) or miR-193a Mimic (M193a) or with a combination of M221 and M193a (12.5 nM each). **Panel A:** Microvessel formation. **Panel B:** wound closure.  $n=3$ ,  $*p<0.05$  compared to control.

### 5.2.6 Discussion

The first aim of this part of the study was to confirm the inhibitory role of miR-221 on HUVEC vasculogenesis, migration and proliferation and to investigate the effect of miR-193a on EC function. Based on the evidence of an anti-proliferative role of miR-193a in several cancer cells [238-247], and the fact that E2 down-regulates the expression of both miR-193a and miR-221, we assumed that miR-193a has comparable physiological effects as miR-221 and that these are opposite to the actions of E2. We further hypothesized that down-regulation of miR-193a and miR-

221 by E2 mediates its stimulatory effects on EC proliferation, migration and vasculogenesis.

Indeed, we confirmed that miR-221 increases EC dysfunction and provide the first evidence for a role of miR-193a in vascular cells. Similar to miR-221 overexpression, the up-regulation of miR-193a by the specific Mimic impaired HUVEC activity, reducing tube formation in the matrigel assay, wound closure and proliferation. The role of miR-193a and miR-221 in ECs is further supported by the contrasting results obtained following miRNA inhibition, including enhancement of tube formation, wound closure and proliferation. Moreover, down-regulation of both miRNAs promoted HUVEC activity similar to the treatment with E2. Finally, we demonstrate that overexpression of miR-193a and miR-221 abrogates E2-induced vasculogenesis and migration by HVUECs, suggesting that down-regulation of these miRNAs contributes to the protective effects of E2 in ECs.

In order to study the physiological role of the miRNAs we employed miRNA Mimics or Antimirs. Although HUVECs are notoriously difficult to transfect and susceptible to the toxicity of transfection reagents, lipofection yielded very good transfection efficiency with little cell toxicity. Successful transfection of HUVECs was confirmed by the inhibition of GAPDH protein expression using a Positive Control miRNA Mimic specifically targeting GAPDH. These results show that the Mimic is efficiently incorporated into RISC, which is required for inhibiting the expression of the target genes, suggesting that all transfected miRNA mimics may be functionally active. We further used RT-qPCR to confirm the uptake of the Mimics and Antimirs and their maintenance in the cells for the time of the longest assay. The level of both miRNAs was dramatically increased by the Mimics; however, this might give a false impression about miRNA levels as location and functionality of the miRNA cannot be evaluated by RT-qPCR. Although RT-qPCR is widely used to test miRNA inhibition by the Antimirs, it might also be problematic, as the antisense can inhibit the qPCR reaction. Moreover, inhibition of miRNA activity has been shown to occur without degradation of the miRNA [252, 253]. We found that miR-221 was significantly reduced after transfection with the Antimir, while miR-193 was not. Nevertheless, miR-193a Antimir stimulated HUVEC function in the biological assays similar to miR-221 Antimir, suggesting that miRNA activity is successfully inhibited though without

changes in miRNA levels. However, off-target effects of the miRNAs cannot be excluded.

We demonstrated that miR-221 decreases tube formation, wound closure and proliferation in HVUECs. Moreover, we provided the first evidence that miR-193a is involved in the regulation of EC functions. Indeed, miR-193a overexpression inhibited vasculogenesis, migration and proliferation of HUVECs similar to miR-221. Accordingly, inhibition of both miRNAs using an Antimir stimulated HUVEC functions.

miR-221 is highly expressed in HUVECs [254], suggesting a critical role of this miRNA in the regulation of EC function. Although most of the studies support our findings that miR-221 inhibits proliferation, migration and vasculogenesis of ECs [215, 221], the anti-angiogenic properties of miR-221 are restricted to mature human ECs. Indeed, in embryonic ECs miR-221 induces angiogenesis and promotes proliferation and migration of tip ECs in sprouting neovessels [255]. Moreover, in miR-221-deficient zebrafish embryos, proliferation of ECs is blocked [256], suggesting that the role of miR-221 greatly varies depending on the developmental stages and microenvironment. Since the function of miR-193a has never been investigated in association of vascular cells or angiogenesis, our findings that miR-193a regulates EC functions, including tube formation, are novel and of potential clinical relevance.

Proliferation, migration and vasculogenesis are all processes, which are influenced by cell viability, and their inhibition may result from increased apoptosis. Therefore, we further investigated the effect of miR-193a and miR-221 on HUVEC survival. The impact of miR-221 on EC survival is controversial. Previous research has demonstrated that miR-221/222 induces EC apoptosis. Liu et al. demonstrated that miR-221/222 inhibits proliferation, migration and cell survival in serum deprived condition [230]. Moreover, miR-221/222 has been reported to exert anti-angiogenic, anti-inflammatory and pro-apoptotic effects in HUVECs by direct targeting of several genes including c-Kit [215] and Ets-1 [257] and indirectly reducing eNOS expression [258]. In contrast, Qin et al. proposed an anti-apoptotic role for miR-221/222 in ECs. Indeed, they showed that miR-221/222 is significantly down-regulated in HUVECs exposed to ox-LDL [259] and that miR-221/222 overexpression decreases ox-LDL-induced HUVEC apoptosis, while miR-221/222 inhibition increased it [260]. In support of these results, overexpression of miR-221/222 has been shown to be associated



with several human cancers, including glioblastoma, hepatocellular carcinoma, breast, prostate, pancreatic and gastric cancer. In tumor cells, miR-221/222 regulated cell survival by directly targeting cell cycle regulators, including p27 and p57, tumor suppressor genes, i.e. PTEN, transcription factors and several pro-apoptotic proteins such as Puma and Bim [261]. The role of miR-193a for EC survival has not yet been investigated; nevertheless, its pro-apoptotic functions are reported in several tumors, including melanoma, hepatocellular carcinoma, acute myeloid leukemia, breast and prostate cancer [245]. Overexpression of miR-193a and miR-193b induced activation of caspase 3/7 and resulted in apoptotic cell death of ovarian cancer cells [238, 262-264]. In this study, we could not detect significant changes in cell viability after modulation of miR-193a or miR-221, suggesting that they do not affect apoptosis in ECs. However, we used indirect methods to study the effect of the miRNAs on cell survival and did not specifically measure apoptosis. Therefore, the analysis of apoptosis using a specific marker, such as Annexin V or TUNEL staining, might give a better answer on how miR-193a and miR-221 influence EC function. Finally, the incongruity about the role of miR-221 on cell viability indicates that the activity of miRNAs might strongly depend on the cell type and cellular environment, thus it is feasible that, under our specific condition, miR-221 and miR-193a do not affect cell survival.

Endothelial damage and dysfunction are part of the underlying causes for atherosclerosis and CVD. E2 exerts its protective effects by promoting EC vasculogenesis, migration and proliferation, thus showing the same outcome as inhibition of miR-193a and miR-221. Indeed, re-expression of the miRNA after down-regulation by E2 treatment abrogated E2-induced tube formation and promotion of wound closure. These findings suggest that down-regulation of miR-193a and miR-221 by E2 participates in the E2-mediated stimulatory effects on HUVECs. Moreover, we observed no additive induction of tube formation after E2 stimulation and simultaneous inhibition of the miRNAs by the Antimirs. These findings support our hypothesis and indicate that the down-regulation of either miR-193a or miR-221 by E2 may be sufficient to abrogate the anti-angiogenic effects of these two miRNAs.

The mechanisms by which E2 supports EC functions include several intracellular pathways. We postulate that miR-193a and miR-221 modulate molecules downstream of E2 and thus mediate its actions on ECs function. Since miRNAs

target numerous mRNAs in multiple pathways, it is possible that miR-193a and miR-221 regulate EC function via the same or different pathways. To test this we overexpressed both miRNAs simultaneously and observed their effect in the matrigel tube formation assay and scratch wound closure assay. We found no additive inhibitory effect when the two Mimics were combined compared to the individual miRNAs, suggesting that miR-193a and miR-221 potentially target the same genes or different genes in the same pathway. However, the outcomes of these experiments should be interpreted with caution, as compared to cells treated with individual miRNAs Mimics, we did not assess their uptake and activity in cells following combined treatment. Several factors may be responsible for the lack of additive effect observed and could and should be experimentally verified as follows. First, the possibility that only one miRNA is efficiently transfected into the cells can be easily tested using RT-qPCR analysis. Second, since we employed only 12.5 nM of each miRNA for the co-transfection, and 25 nM of each miRNA alone, it is possible that the additive effect on the stimulation of HUVEC tube formation and wound closure were masked. Therefore, the effects of 12.5 nM of each miRNA Mimics on the function of HUVECs needs to be examined to exclude concentration dependent effects. A third issue may be the competition between the co-administered miRNAs for the binding of AGO2, the limiting component of the RISC complex, which may be responsible for the absence of additive effects [265]. Hence, it is recommended to confirm the functional activation of both miR-193a and miR-221 Mimics. The activity of the miRNAs can be verified using luciferase or GFP reporters, designed with a 3'UTR sequence, which are targeted by either one or the other miRNA, and comparing the output after transfection with negative control, miR-193a Mimic and miR-221 alone and the combination of both Mimics. Another experiment to demonstrate the presence of the miRNAs in the RISC complex would be to isolate the overall small RNAs bound to AGO2 using RNA binding immunoprecipitation (RIP), and examine the presence of miR-193a and miR-221 by RT-qPCR [60, 266].

In conclusion, we propose that miR-193a and miR-221 down-regulation by E2 suppresses the inhibitory actions of miR-193a and miR-221 on proliferation, migration and vasculogenesis, thus imparting a promoting role for E2 on these same processes. Moreover, we speculate that miR-193a and miR-221 modulate mRNAs of genes within the same pathways, which are important for E2-mediated endothelial protection.

### 5.3 Intracellular mechanisms of E2-regulated miRNAs in ECs

#### Objective

Our findings in HUVECs provide evidence that down-regulation of miR-193a and miR-221 mediates the growth and vasculogenic effects of E2 and may contribute to its vaso-protective actions. Hence, our next aim was to investigate the underlying intracellular mechanisms potentially involved. The PI3K/ Akt pathway, VEGF-A and the ALK1/ SMAD1/5/8/ Id1 pathway are among important E2-regulated pathways in vasculogenesis; therefore, we assessed whether they are modulated by miR-193a and miR-221.

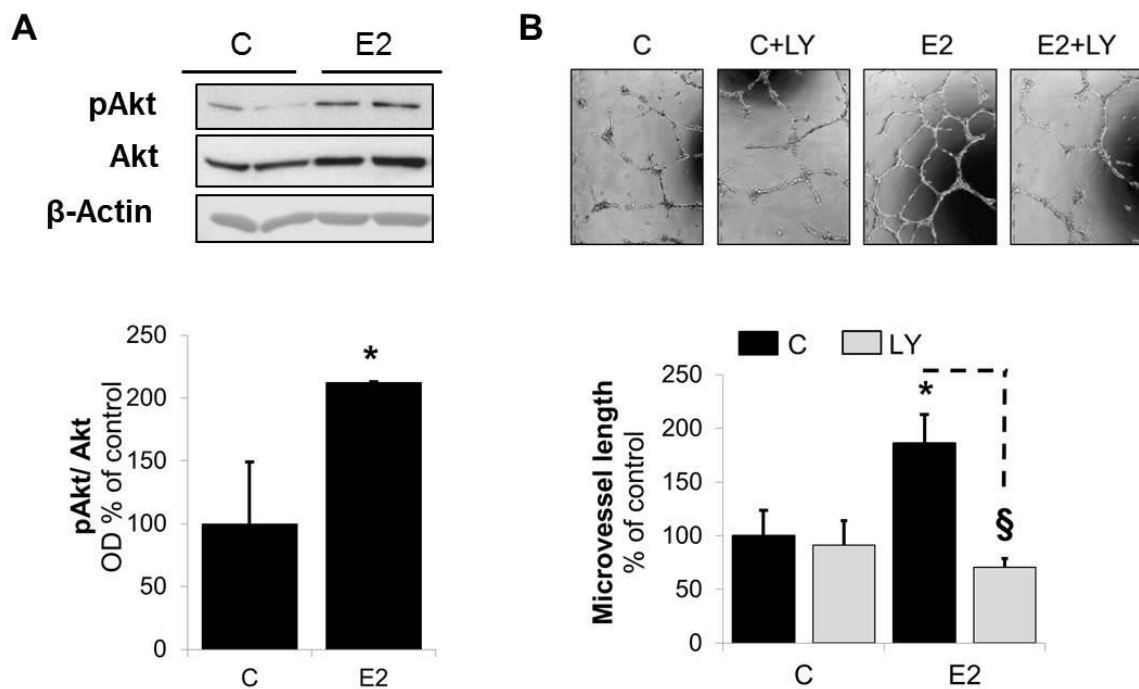
#### Introduction

In the previous section (5.2) we demonstrated that miR-193a and miR-221 inhibit proliferation, wound closure and tube formation in HUVECs. Moreover, overexpression of the individual miRNAs abolished the stimulatory effects of E2 on HUVEC function, suggesting that E2 induces vasculogenesis and migration of ECs by down-regulating the expression of miR-193a and miR-221. Furthermore, combined treatment with miR-193a and miR-221 Mimics did not enhance the inhibitory effects seen with the individual miRNAs on tube formation and wound closure, suggesting that they potentially target the same intracellular pathway.

E2 stimulates EC proliferation, migration and tube formation via activation of PI3K/ Akt signaling [136, 151-153]. Moreover, E2-induced angiogenesis is dependent on VEGF [135, 154]. Another angiogenic pathway involved is the ALK1/ SMAD1/5/8/ Id1 signaling pathway [170, 171]. Based on our findings that down-regulation of miR-193a and miR-221 by E2 potentially mediates its vasculogenic actions in ECs, we postulate that they have an inhibitory role on one or more of these signaling pathways. We therefore investigated whether miR-193a and miR-221 modulate PI3K/ Akt signaling, VEGF and/ or ALK1/ SMAD1/5/8/ Id1 signaling and whether these pathways contribute to the modulatory actions of miR-193 and miR-221 on vasculogenesis.

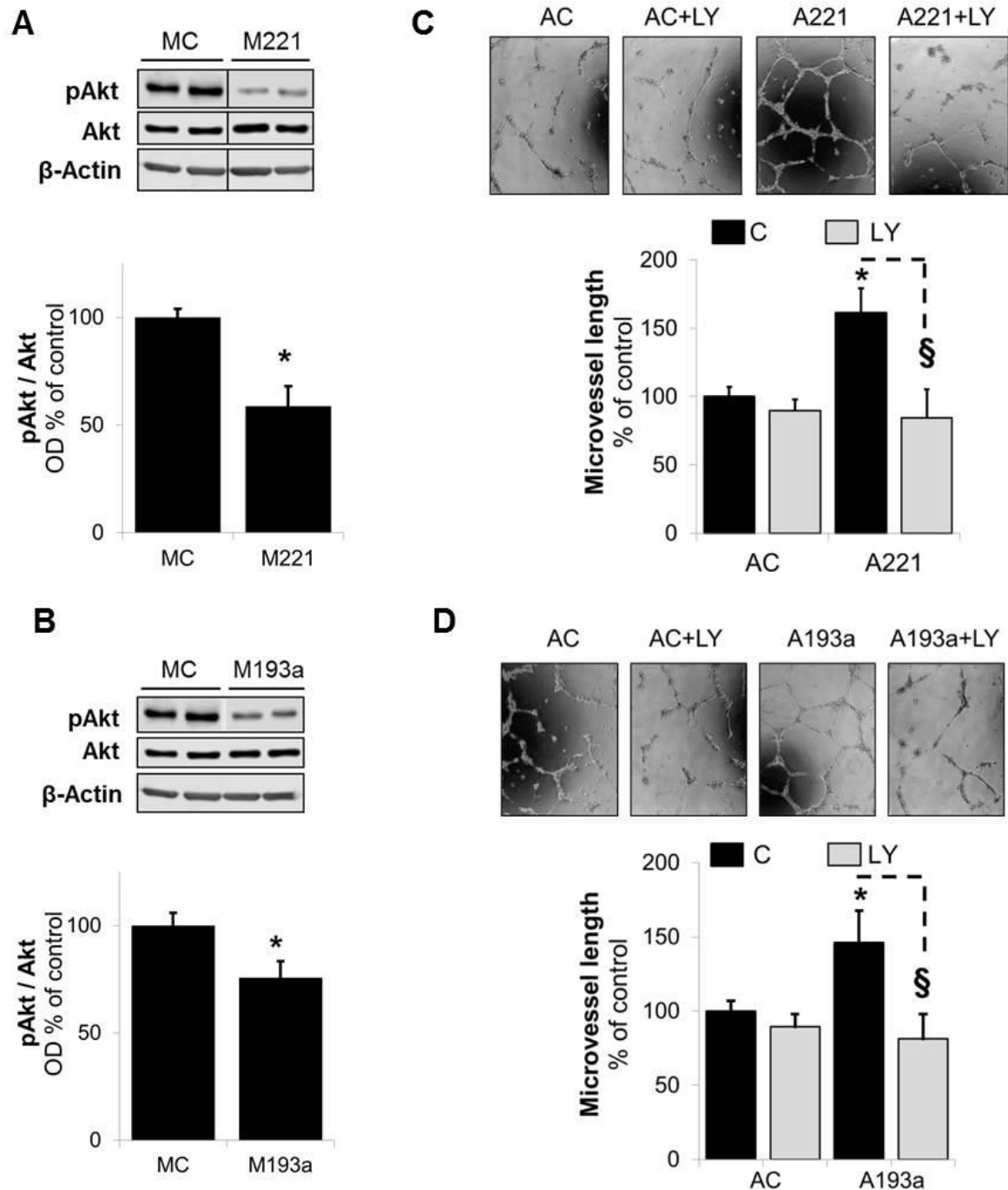
### 5.3.1 The role of the PI3K/ Akt pathway in mediating the effects of E2 and miR-193a and miR-221 in vasculogenesis

The PI3K/ Akt pathway is a recognized pro-angiogenic pathway and plays a role in mediating several actions of E2 in different cell types, including ECs. We first confirmed that treatment of HUVECs with E2 (10 nM) increased Akt phosphorylation from  $100\pm 49\%$  to  $212\pm 1\%$  ( $p<0.05$ ; **Figure 31A**). Next, we used the pharmacological PI3K inhibitor LY294002 (LY; 5  $\mu\text{M}$ ) to assess the role of E2-induced Akt signaling in mediating vasculogenesis in HUVEC. Indeed, we found that LY significantly abrogated the stimulatory effect of E2 from  $186\pm 27\%$  to  $70\pm 8\%$  (**Figure 31B**), thus confirming that activation of the PI3K/ Akt pathway is important for E2-induced microvessel formation.



**Figure 31: E2 induces vasculogenesis via the PI3K/ Akt pathway. Panel A:** Representative Western blots and bar graphs for Akt phosphorylation (pAkt, Ser473) after treatment with 10 nM E2 for 45 minutes. Total Akt and  $\beta$ -actin were used as loading controls. **Panel B:** The PI3K inhibitor LY294002 (LY, 5  $\mu\text{M}$ ) abrogates the inducing effect of E2 on tube formation. HUVECs were pre-treated for 30 minutes with LY and subsequently for 30 minutes with 10 nM E2 and plated on the matrigel. Tube length was measured after 16-18 hr. Photomicrographs show representative images for each treatment.  $n=3$ , \* $p<0.05$  compared to the respective control, § $p<0.05$  as indicated.

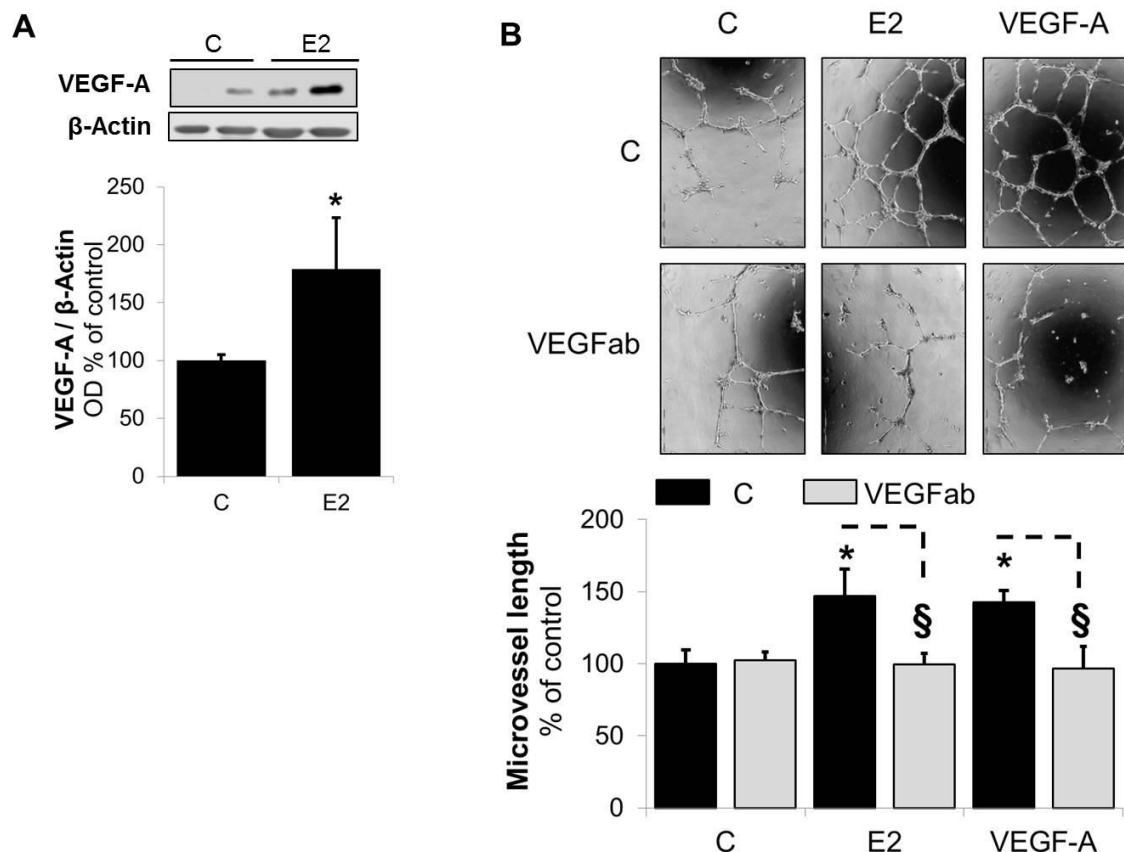
To investigate the role of PI3K/ Akt in miR-221 and miR-193a mediated inhibition of tube formation, we examined whether their overexpression was able to produce changes in Akt phosphorylation. Indeed, we found that both miR-221 and miR-193a Mimics significantly reduced Akt phosphorylation from  $100\pm6\%$  to  $59\pm8\%$  and to  $75\pm8\%$ , respectively (**Figure 32A and 32B**). To further confirm the contribution of PI3K/ Akt as part of the mechanism, we assessed the microvessel formation in the presence or absence of the PI3K inhibitor LY after down-regulation of the miRNAs by the respective Antimirs (**Figure 32C and 32D**). Interestingly, the stimulatory effect produced by A221 through down-regulation of the anti-angiogenic miR-221 is abrogated by treatment of the cells with LY: from  $161\pm18\%$  to  $84\pm21\%$  ( $p<0.05$ ). We found similar results by combining transfection of the cells with Antimir A193a and LY treatment: microvessel formation was reduced from  $146\pm22\%$  to  $81\pm17\%$  ( $p<0.05$ ). These results suggest a role for the PI3K/ Akt pathway in the regulation of vasculogenesis by miR-193a and miR-221.



**Figure 32: miR-221 and miR-193a inhibit vasculogenesis via the PI3K/ Akt pathway.** **Panel A and B:** Representative Western blots and bar graphs for Akt phosphorylation (pAkt, Ser473) after transfection with Control Mimic (MC), miR-221 Mimic (M221) or miR-193a Mimic (M193a). Total Akt and  $\beta$ -actin were used as loading controls. **Panel C and D:** The PI3K inhibitor LY294002 (LY, 5  $\mu$ M) abrogates the stimulatory effects of the Antimirs on tube formation. Cells were transfected with Control Antimir (AC) or Antimirs for miR-221 (A221) or miR-193a (A193a) and treated 30 minutes with LY prior to being plated on matrigel. Tube length was measured after 16-18 hr. Photomicrographs show representative images for each treatment. n=3, \*p<0.05 compared to the respective control, §p<0.05 as indicated.

### 5.3.2 The role of VEGF-A in mediating the effects of E2 and miR-193a and miR-221 in vasculogenesis

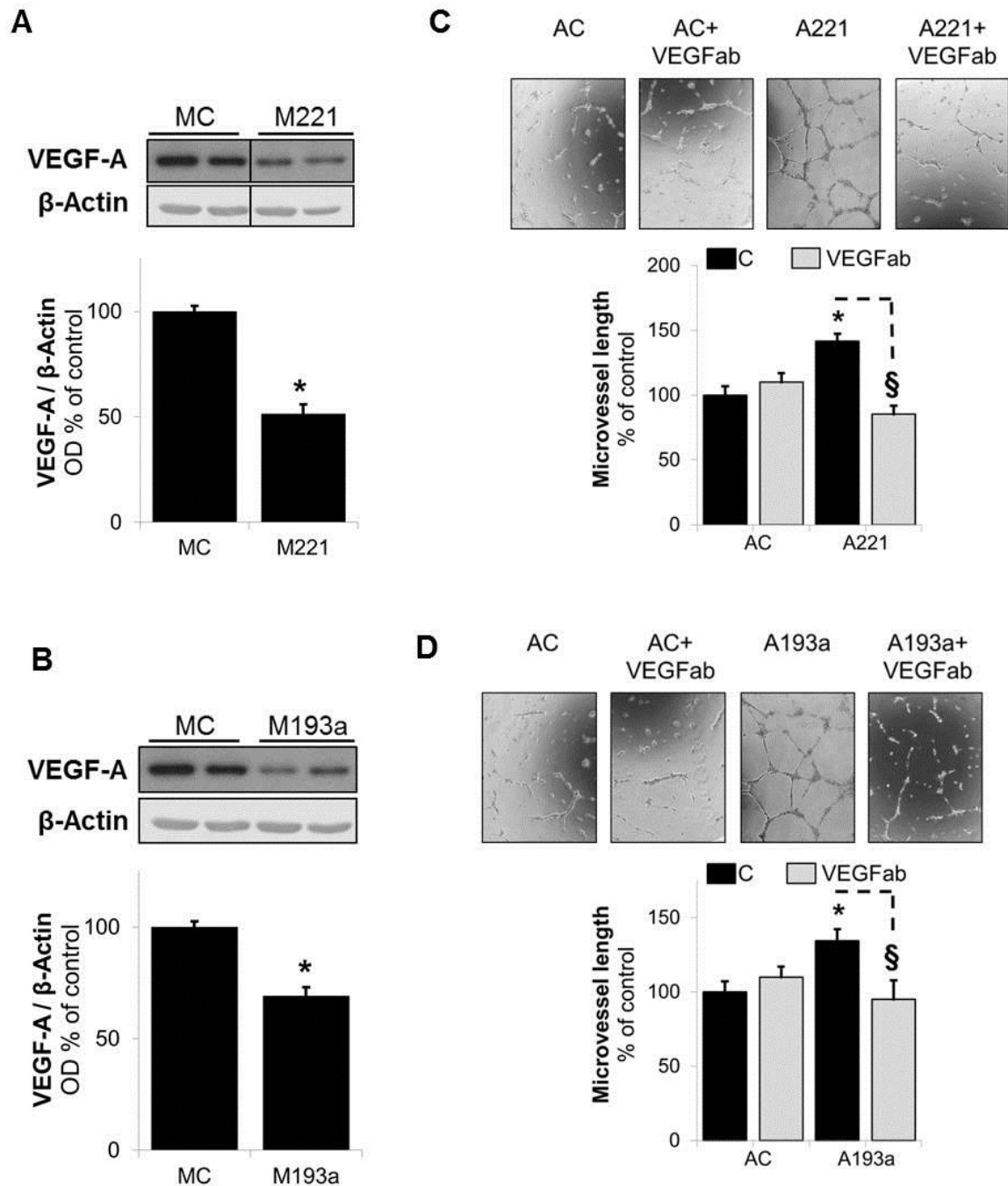
The pro-angiogenic properties of E2 are also due to induction of VEGF-A, whose expression was significantly increased following 45 minutes E2-treatment from  $100 \pm 4\%$  to  $179 \pm 45\%$  ( $p < 0.05$ ; **Figure 33A**). Moreover, microvessel formation was abrogated when the cells were pre-treated with a neutralizing antibody for VEGF-A, from  $147 \pm 19$  to  $99 \pm 8\%$  ( $p < 0.05$ ; **Figure 33B**). As a positive control, HUVECs were treated with VEGF-A alone or in combination with its neutralizing antibody. VEGF-A significantly induced microvessel formation by from  $100 \pm 10\%$  to  $142 \pm 8\%$  and this stimulatory effect was inhibited by the VEGF-A neutralizing antibody (from  $142 \pm 8\%$  to  $97 \pm 15\%$ ,  $p < 0.05$ ).



**Figure 33: E2 dependent vasculogenesis involves VEGF-A.** **Panel A:** Representative Western blot and bar graph of VEGF-A protein expression after 45 minutes treatment with 10 nM E2).  $\beta$ -Actin was used as loading control. **Panel B:** The VEGF-A specific neutralizing antibody (VEGFab, 500 ng/ml) abrogates the stimulatory effects of E2 and VEGF-A on tube formation. HUVECs were pre-treated for 30 minutes with VEGFab before a 30 minute treatment with E2 (10 nM) or VEGF-A (100 ng/ml). Cells were plated on matrigel, and tube length was measured after 16-18 hr. Photomicrographs show representative images for each treatment.  $n=3$ , \* $p < 0.05$  compared to the respective control, § $p < 0.05$  as indicated.

To assess the role of VEGF-A in miR-221 and miR-193a mediated inhibition of microvessel formation, we examined the effect of miRNA Mimics on VEGF-A expression in HUVECs. Both miR-221 and miR-193a significantly reduced VEGF-A expression from  $100\pm3\%$  to  $51\pm5\%$  and  $69\pm4\%$ , respectively ( $p<0.05$ ; **Figure 34A and 34B**). In agreement with these actions, treatment with the Antimirs induced tube formation and this effect was blocked in the presence of the VEGF-A neutralizing antibody (**Figure 34C and 34D**). A221-induced microvessel formation was decreased from  $142\pm6\%$  to  $85\pm7\%$  ( $p<0.05$ ), while VEGFab inhibited A193a-induced tube formation from  $134\pm8\%$  to  $95\pm13\%$  ( $p<0.05$ ). These results suggest that VEGF-A plays a role in the regulation of vasculogenesis by miR-193a and miR-221.

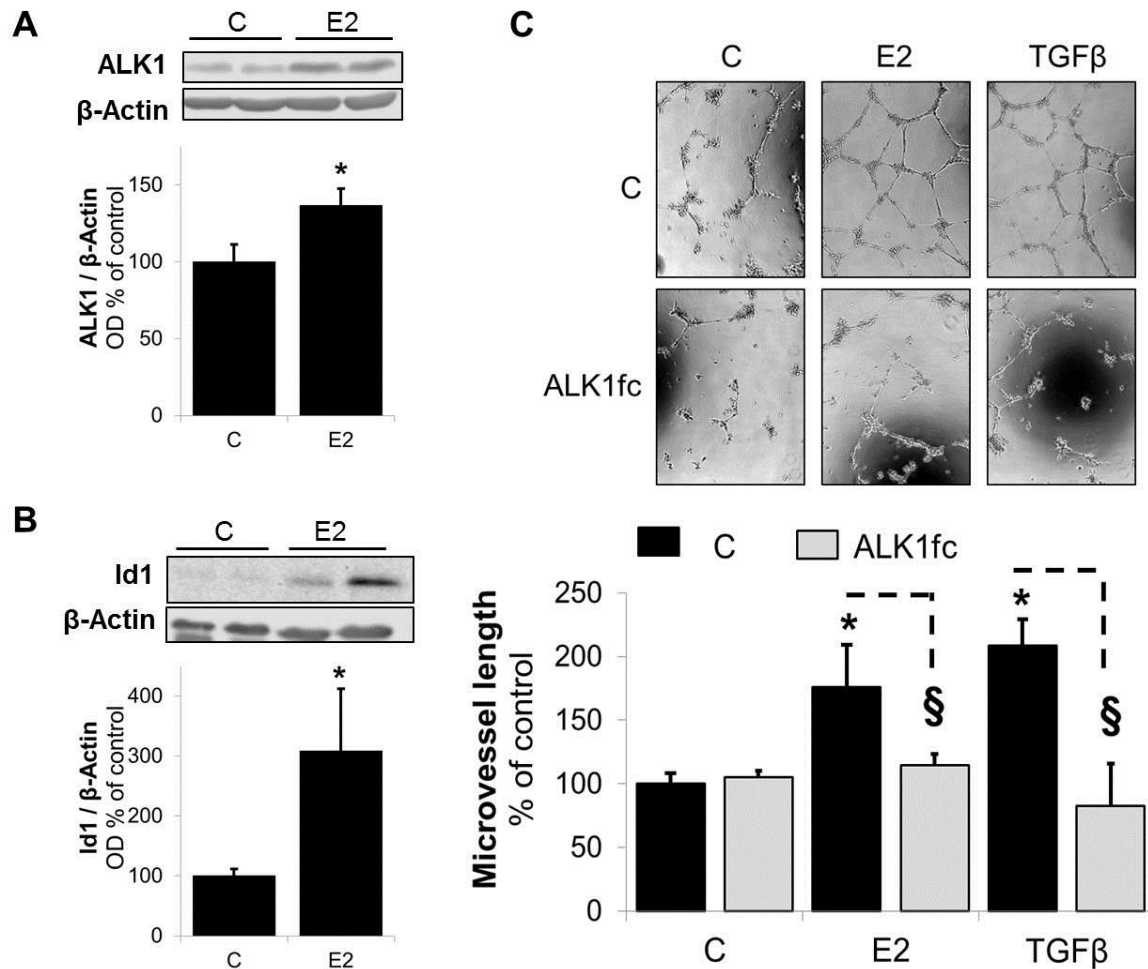




**Figure 34: miR-221 and miR-193a inhibit vasculogenesis via VEGF-A. Panel A and B:** Representative Western blots and bar graphs of VEGF-A protein expression after transfection of the cells with Control Mimic (MC), miR-221 Mimic (M221) or miR-193a Mimic (M193a).  $\beta$ -Actin was used as loading control. **Panel C and D:** The VEGF-A neutralizing antibody (VEGFab, 500 ng/ml) abrogates the inducing effects of the Antimirs on tube formation. HUVECs were transfected with Control Antimir (AC), miR-221 Antimir (A221) or miR-193a Antimir (A193a) and left in growing media for 24 hr before 30 minutes treatment with VEGFab. Cells were plated on matrigel, and tube length was measured after 16-18 hr. Photomicrographs show representative images for each treatment.  $n=3$ , \* $p<0.05$  compared to the respective control, § $p<0.05$  as indicated.

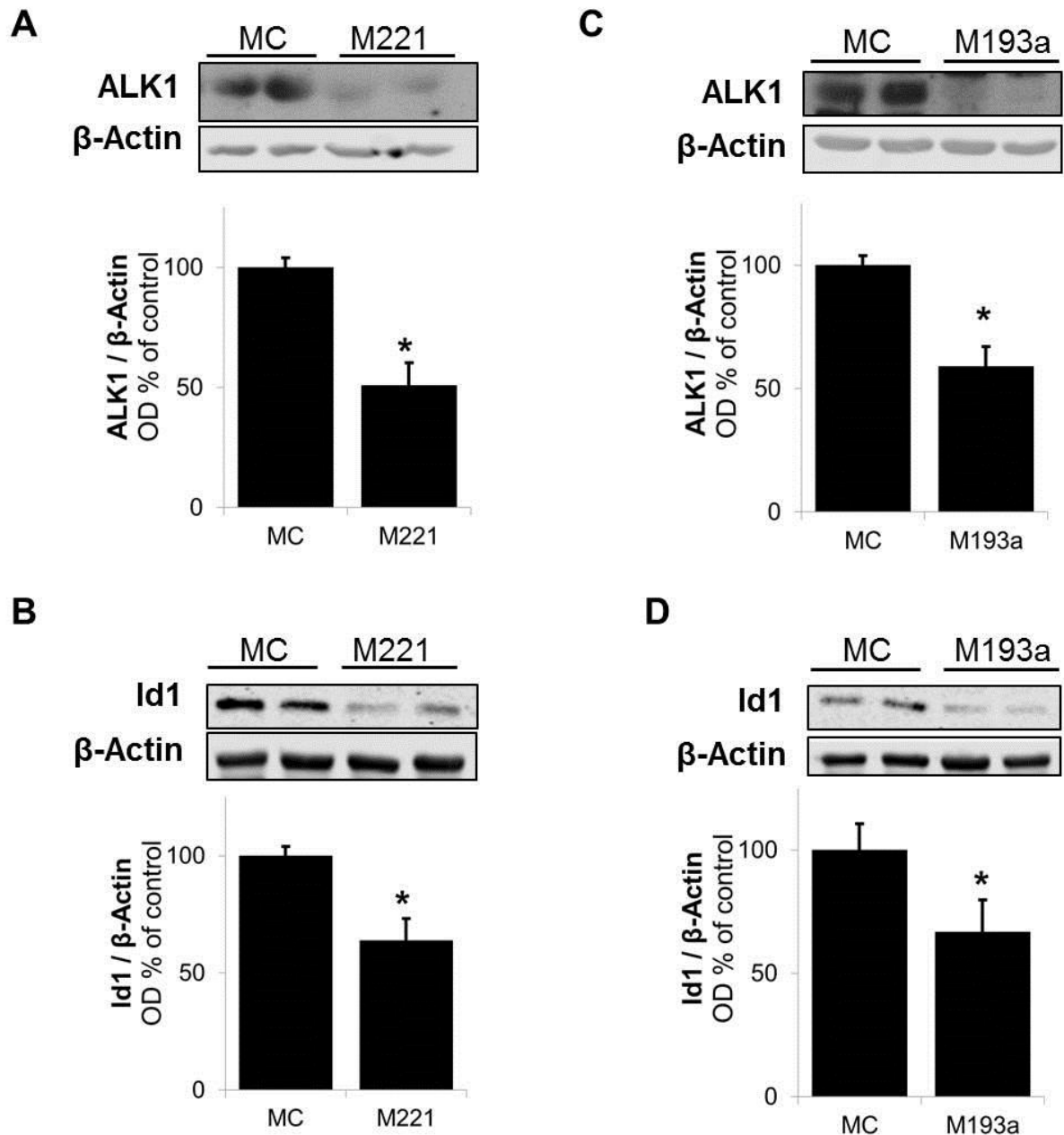
### 5.3.3 The role of ALK1/ SMAD1/5/8/ Id1 pathway in mediating the effects of E2 and miR-193a and miR-221 in vasculogenesis

E2 is known to induce microvessel formation via the ALK1/ SMAD1/5/8/ Id1 pathway. Activation of ALK1 leads to SMAD1/5/8 phosphorylation, which in turn leads to increased expression of its downstream target Id1, the protein responsible for inducing migration and tube formation in ECs. In HUVECs treated with E2 we found increased expression of ALK1 protein (from  $100\pm 11\%$  to  $137\pm 11\%$ ,  $p<0.05$ ; **Figure 35A**) as well as a significant induction of Id1 from  $100\pm 13\%$  to  $309\pm 11\%$  (**Figure 35B**). The role of the ALK1/ SMAD1/5/8/ Id1 pathway was further confirmed by the fact that Alk1fc, a specific antagonizing antibody for ALK1, abrogated E2-induced microvessel formation (from  $176\pm 33\%$  to  $115\pm 8\%$ ,  $p<0.05$ ). Stimulation by TGF $\beta$  was used as a positive control, since it is well documented to activate the ALK1/ SMAD1/5/8 pathway. Indeed, Alk1fc repressed TGF $\beta$  induced microvessel formation from  $209\pm 20\%$  to  $82\pm 33\%$  ( $p<0.05$ ; **Figure 35C**).



**Figure 35: E2 induces vasculogenesis via the ALK1/ Id1 pathway. Panel A and B:** Representative Western blots and bar graphs of ALK1 and Id1 protein expression after 45 minutes treatment with 10 nM E2.  $\beta$ -Actin was used as loading control. **Panel C:** ALK1 specific antagonizing antibody (ALK1fc, 100 ng/ml) abrogates the effects of E2 and TGF $\beta$  on tube formation. HUVECs were pre-treated 30 minutes with ALK1fc before 30 minutes treatment with E2 (10 nM) or TGF $\beta$ 1 (1 ng/ml) and plated on matrigel. Tube length was measured after 16-18 hr. Photomicrographs show representative images for each treatment. n=3, \*p<0.05 compared to the respective control, §p<0.05 as indicated.

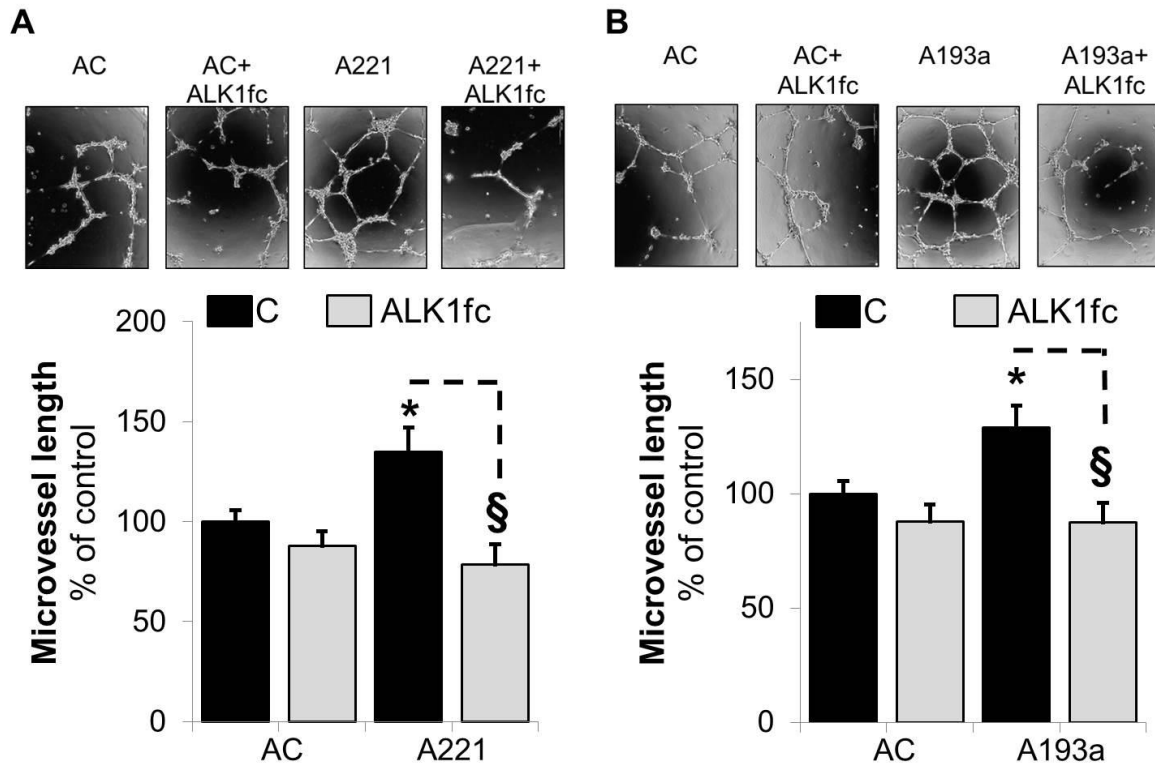
Since we were interested in assessing whether miR-221 and miR-193a mediate the effects of E2 in ECs, we examined their effect on the ALK1/ SMAD1/5/8/ Id1 pathway. We found that overexpression of miR-221 by its Mimic M221 repressed ALK1 expression (from 100 $\pm$ 4% to 51 $\pm$ 9%, p<0.05) as well as the downstream target Id1 (from 100 $\pm$ 11% to 64 $\pm$ 9%, p<0.05; **Figure 36A and 36B**). Similar to miR-221, treatment with miR-193a Mimic (M193a) down-regulated ALK1 and Id1 expression from 100 $\pm$ 10% to 59 $\pm$ 8% and to 67 $\pm$ 13%, respectively (p<0.05; **Figure 36C and 36D**).



**Figure 36: miR-221 and miR-193a inhibit the ALK1/ Id1 pathway.** The representative Western blots and bar graphs of ALK1 (**Panel A and C**) and Id1 (**Panel B and D**) protein expression after transfection of the cells with Control Mimic (MC), miR-221 Mimic (M221) or miR-193a Mimic (M193a).  $\beta$ -Actin was used as loading control.  $n=3$ , \* $p<0.05$  compared to control.

To further confirm the involvement of the ALK1/ SMAD1/5/8/ Id1 pathway in the effect of the miRNAs on E2-induced vasculogenesis, we assessed microvessel formation following simultaneous treatment of the cells with the ALK1 inhibitor Alk1fc and transfection with the Antimirs (**Figure 37A and 37B**). The stimulatory effects of A221 (from  $100\pm6\%$  to  $135\pm12\%$ ,  $p<0.05$ ) and A193a (from  $100\pm6\%$  to  $129\pm9\%$ ,  $p<0.05$ )

were significantly abrogated in both cases by ALK1fc by  $57\pm 10\%$  and  $42\pm 8\%$ , respectively, suggesting that this pathway actively contributes to the regulation of microvessel formation by the two miRNAs.



**Figure 37: miR-221 and miR-193a inhibit vasculogenesis via the ALK1/ Id1 pathway.** The ALK1 antagonizing antibody (ALK1fc, 100 ng/ml) abrogates the inducing effects of the Antimirs on tube formation. HUVECs were transfected with Control Antimir (AC), miR-221 Antimir (A221; **Panel A**) or miR-193a Antimir (A193a; **Panel B**) and left in growing media for 24 hr before 30 minutes treatment with ALK1fc. Cells were plated on matrigel and tube length was measured after 16-18 hr. Photomicrographs show representative images for each treatment.  $n=3$ , \* $p<0.05$  compared to the respective control, § $p<0.05$  as indicated.

### 5.3.4 Discussion

We investigated the effects of miR-193a and miR-221 on PI3K/ Akt signaling, VEGF expression and the ALK1/ SMAD1/5/8/ Id1 pathway, which are known to mediate the stimulatory actions of E2 on EC proliferation, migration and vasculogenesis.

In ECs the PI3K/ Akt signaling axis is activated by a variety of stimuli and regulates multiple critical steps in angiogenesis, including endothelial cell survival, migration, and tube formation. We first investigated whether E2 stimulates PI3K/ Akt signaling in HUVECs and observed an increase in Akt phosphorylation at Serine473 after

treatment with E2. Our findings are consistent with the results from previous studies, which demonstrated that E2 increases phosphorylation of Akt in ECs and that these effects are blocked by the PI3K inhibitors LY294002 and Wortmannin [267, 268]. The importance of PI3K/ Akt signaling in estrogen-mediated angiogenesis is well established [136, 151, 152]. Our findings that E2 induces Akt phosphorylation and tube formation in HUVECs and that these effects are blocked by the PI3K inhibitor LY294002 further reconfirms this contention.

The role of miR-221 in ECs has been extensively studied and we confirmed its anti-angiogenic action in HUVECs. Moreover, we demonstrated that down-regulation of miR-221 potentially mediates the stimulatory actions of E2 on vasculogenesis. Since PI3K/ Akt signaling is required for the pro-angiogenic actions of E2, we investigated whether miR-221 participates in the regulation of this pathway. Here we provide evidence that miR-221 overexpression inhibits the phosphorylation of Akt. Moreover, treatment with LY294002 abrogated tube formation following miR-221 knock-down, suggesting that miR-221 inhibits vasculogenesis by targeting the PI3K/ Akt pathway and may importantly contribute to the vascular repair process.

It has been reported that the anti-angiogenic properties of miR-221 are mediated through the regulation of many targets, including c-Kit, the cell cycle regulator p27, transcription factors Ets1, Ets2 [215], zinc finger E-box binding homeobox 2 (ZEB2) [269], signal activator and transducer 5A (STAT5a) [270] and endothelial NO-synthase [209]. In addition, direct effects of miR-221 on the PI3K/ Akt signaling axis has been demonstrated in embryonic ECs. miR-221 regulates endothelial tip cell migration and proliferation by targeting the PI3K regulatory subunit 1 (PIK3R1) [221]. PI3KR1 is the p85 $\alpha$  regulatory subunit of the PI3K complex and an essential component for PI3K signaling in vascular development [271]. In the absence of stimuli, PIK3R1 stabilizes the catalytic subunit, such as p110, of the PI3K signaling complex and inhibits its kinase activity. However, PIK3R1 is also required for PI3K activity following membrane localization of the PIK3r1/p110 complex and activation by a receptor tyrosine kinase [272]. By targeting PIK3R1, miR-221 is likely to alter an appropriate balance between regulatory and catalytic subunits. Since PI3KR1 both inhibits and is required for PI3K/ Akt activity, it is feasible that miR-221 suppresses angiogenesis in mature human ECs through targeting PIK3R1. However, this assumption needs to be further verified. In contrast to our findings that miR-221

inhibits PI3K/ Akt signaling, miR-221 has been demonstrated to target the negative regulator of the pathway, Phosphatase and Tensin Homolog (PTEN), in several cancer cell types, i.e. osteosarcoma [273], human glioblastoma [274] and gastric carcinoma [275]. Since PTEN suppression would result in increased Akt activity, we speculate that PTEN is not inhibited by miR-221 in HUVECs. Given the fact that the PI3K/ Akt pathway is activated by upstream receptor tyrosine kinases and interacts with other signaling cascades such as MAPK signaling pathways [276, 277], further research is necessary to elucidate the mechanisms behind miR-221 mediated inhibition of Akt activity. In this regard, several studies provide evidence that miR-221 inhibits EC proliferation, migration and tube formation through inhibition of stem cell factor (SCF) receptor c-Kit [223, 230], and that Akt has an essential role downstream of SCF/ c-Kit signaling, as the c-Kit inhibitor STI-571 abrogated Akt phosphorylation and SCF-driven tube formation [278]. Moreover, Khella et al. [279] have shown that expression of VEGF receptor 2 (VEGFR2) is significantly decreased after miR-221 overexpression in HUVECs, suggesting that miR-221 blocks relevant signaling pathways involved in angiogenesis and cell proliferation by targeting KDR (VEGFR2). Hence, these findings suggest that miR-221 might inhibit Akt activity indirectly by suppressing SCF/ c-Kit or VEGF signaling.

Here we provide the first evidence for an inhibitory role of miR-193a in regulating EC functions. Overexpression of miR-193a decreased vasculogenesis, migration and proliferation of ECs and knock-down of the miRNA showed opposite results. Moreover, we demonstrated that the stimulatory actions of E2 on vasculogenesis are potentially mediated via down-regulation of miR-193a. Since PI3K/ Akt signaling is required for the pro-angiogenic actions of E2, we investigated whether miR-193a participates in the regulation of this pathway. Indeed, miR-193a overexpression inhibits the phosphorylation of Akt. Further, treatment with LY294002 abrogated tube formation seen after miR-193a knock-down, suggesting that miR-193a inhibits vasculogenesis by targeting the PI3K/ Akt pathway.

The association of miR-193a and the PI3K/ Akt pathway has been shown in few studies. In acute myeloid leukemia, miR-193a indirectly inhibits PTEN, thus activating Akt and contributing to leukemogenesis [264]. However, this is in contrast of the inhibitory actions we found in HUVECs. Other evidence of miR-193a association with PI3K/ Akt has been shown in non-small-cell lung cancer, where it suppresses its

metastasis by targeting the receptor tyrosine-protein kinase ERBB4, a member of the EGF receptor subfamily, and inhibiting the downstream PI3K/ Akt cascade [280]. These findings suggest that miR-193a actions on PI3K/ Akt signaling depend on the cell type. Moreover, we speculate that miR-193a targets molecules upstream of PI3K, such as ERBB4 or other receptor tyrosine-protein kinases, resulting in the inhibition of PI3K activity. Finally, there is evidence that miR-193a targets c-Kit in myeloid leukemia [262] similar to miR-221, thus it is feasible that miR-193a might inhibit PI3K activity indirectly via suppressing SCF/ c-Kit or VEGF signaling.

The activation of Akt leads to phosphorylation of eNOS and NO production, which contributes to increased vasodilation and stimulation of angiogenesis [141]. NO induces VEGF expression [267, 268], which in turn stimulates the PI3K/ Akt pathway. Hence NO and VEGF stimulate each other in a feedback circle until PI3K signaling is terminated [144-146, 150].

Treatment with E2 has been shown to increase VEGF expression in vascular ECs [154, 281-283]. Moreover, increased VEGF expression has been associated with E2-mediated endothelial recovery [284]. Indeed, both VEGF and NO are important mediators of estrogenic actions in ECs and there is evidence that estrogen induced angiogenesis is dependent on VEGF and NO [135]. Here we confirmed that treatment with E2 increases VEGF-A expression. In addition, treatment with a VEGF specific neutralizing antibody (VEGFab) abrogated E2-induced vasculogenesis in HUVECs, suggesting that VEGF plays indeed an important role in mediating the stimulatory actions of E2 in ECs.

In the current study we show that, in contrast to E2, miR-221 and miR-193a up-regulation inhibits VEGF-A expression. Furthermore, our observations that vasculogenesis induced by the Antimirs neutralizing miR-221 and miR-193a is abrogated by VEGFab, suggest that VEGF plays a role in the regulation of tube formation by miR-221 and miR-193a in HUVECs. Recently, miR-221 has been shown to inhibit the release of NO by inhibiting eNOS expression [209]. Since NO up-regulates VEGF expression, it is possible that the decrease in VEGF expression is due to inhibition of eNOS as well as the suppression of PI3K/ Akt/ eNOS axis. With regard to miR-193a, Hua et al. found miR-193a binding sites in the VEGF 3'-UTR, thus predicting that miR-193 could putatively target VEGF, based on bioinformatics algorithms. However, miR-193a was not differentially expressed in hypoxic compared



to control nasopharyngeal carcinoma cells, and therefore the association between miR-193a and VEGF was not further investigated [285]. Here we demonstrate that miR-193a inhibits VEGF; however, whether this occurs via direct binding to its mRNA or via down-regulation of PI3K/ Akt signaling pathway remains unclear and needs to be further investigated. The fact that PI3K/ Akt induces eNOS, which in turn up-regulates VEGF via NO, supports that the inhibitory actions of miR-193a and miR-221 on PI3K/ Akt may contribute to their inhibitory effects on VEGF and thus tube formation. Indeed, inhibition of PI3K/ Akt by LY blocks NO release, VEGF expression and angiogenesis [286, 287] and this may contribute to its abrogating actions on Antimir induced capillary formation.

Estrogens regulate TGF $\beta$  signaling [169], another prominent pathway in the regulation of EC growth, migration and angiogenesis [288]. E2 increased the expression of ALK1 and Id1, a downstream target of SMAD1/5/8. Moreover, the ALK1 specific antagonizing antibody ALK1fc abrogated E2-induced vasculogenesis, confirming previous findings from our laboratory demonstrating that E2 mediates vasculogenesis and endothelial growth via the stimulation of ALK1/ Id1 signaling [170, 171].

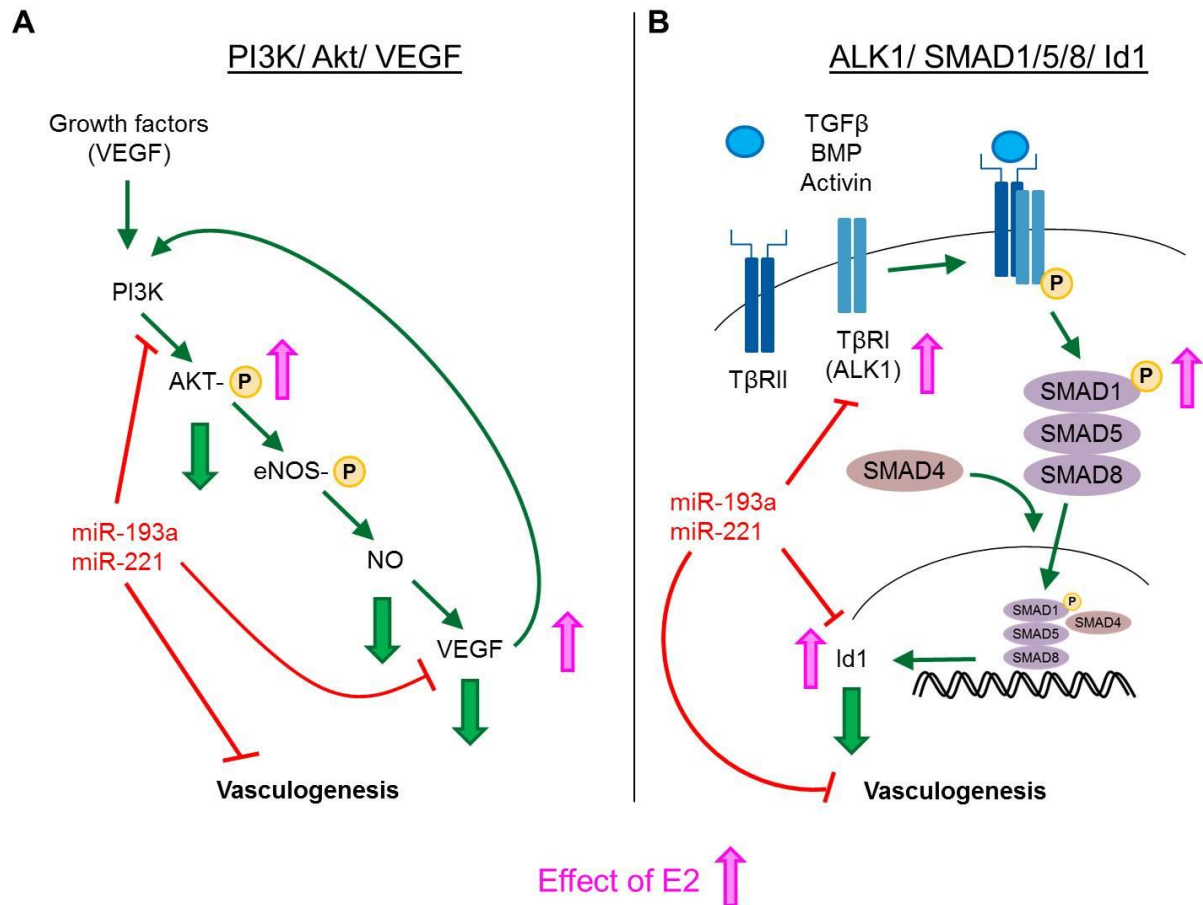
In the current study we provide the first evidence that miR-221 inhibits ALK1 and Id1. We demonstrate that neutralization of miR-221 by its Antimir stimulates vasculogenesis, suggesting that the pro-angiogenic actions of ALK1/ Id1 are suppressed by miR-221. This notion is supported by the sole evidence provided by Rao et al., who reported an association between miR-221 and TGF $\beta$  signaling in breast cancer cells. In this context, overexpression of miR-221/222 was associated with increase of TGFBR2, BMPR2, SMAD1/2/3, Id1 and Id3 expression. Based on their findings, Rao et al. conclude that, although it is not clear whether the pathway is activated or repressed, the growth inhibitory effects of TGF $\beta$  are suppressed by miR-221/222 [92].

The role miR-193a in association with TGF $\beta$  has not been reported. Here we provide the first evidence that overexpression of miR-193 inhibits the expression of both ALK1 and Id1, the downstream target of SMAD1/5/8. Moreover, our finding that neutralization of miR-193a with its Antimir induces vasculogenesis and that these pro-angiogenic actions are blocked in the presence of ALK1fc provide evidence that ALK1/ Id1 signaling is involved in the inhibitory actions of miR-193a on

vasculogenesis. Taken together, these findings suggest that miR-193 decreases vasculogenesis via down-regulation of the pro-angiogenic ALK1/ Id1 signaling. Although there is no direct evidence for a role of miR-193a in this pathway, our findings are supported by the fact that, based on bioinformatics algorithms such as TargetScan [46] and miRIAD [289], ALK1 is a putative target of miR-193a.

Interestingly, miR-221 and miR-193a showed similar inhibitory effects on all the tested pathway proteins, though they do not share the same seed sequence, required for pairing to complementary sites within target mRNAs. However, this evidence does not exclude that the two miRNA could bind to a same target, although at different locations.

In summary, we confirmed that E2 induces vasculogenesis via the PI3K/ Akt pathway, through stimulation of VEGF, and via ALK1/ Id1 signaling. Moreover, we provide the first evidence that miR-221 and miR-193a inhibit PI3K activity, VEGF expression and both ALK1 and Id1 expression in HUVECs. Further, as outlined in **Figure 38**, we demonstrated that all the investigated pathways are involved in miR-221 and miR-193a mediated inhibition of vasculogenesis, as tube formation after miRNA down-regulation was abrogated by the PI3K inhibitor LY294002, the VEGF neutralizing antibody VEGFab and the ALK1 specific antagonizing antibody ALK1fc. In addition to the role in angiogenic processes, these pathways are also important for EC migration and proliferation. Therefore, based on our results, we postulate that the stimulatory effects of E2 on the endothelium is in part mediated via the down-regulation of miR-221 and miR-193a and the suppression of their inhibitory action on PI3K/ Akt, VEGF and ALK1/ Id1. However, the mechanism(s) by which miR-221 and miR-193a regulate these signaling pathways need to be further elucidated in order to provide a better understanding of miRNA mediated E2-induced endothelial protection.



**Figure 38: Summary of our findings in a simplified schematic representation of the pro-vasculogenesis pathways PI3K/ Akt/ VEGF (A) and ALK1/ SMAD1/5/8/ Id1 (B) and their regulation by E2.** *PI3K*, Phosphoinositide 3 Kinase; *Akt*, Protein kinase B; *VEGF*, Vascular Endothelial Growth Factor; *eNOS*, endothelial Nitric Oxide Synthase; *NO*, Nitric Oxide; *ALK1*, Activin Receptor-Like Kinase1; *TGFβ*, Transforming Growth Factor β; *BMP*, Bone Morphogenetic Protein; *TβR*, TGFβ Receptor; *Id1*, Inhibitor of Differentiation.

## 5.4 E2 regulates miRNAs in SMCs

### Objective

E2 protects the vasculature by promoting endothelial integrity and function as well as by inhibiting vascular SMC proliferation and migration. However, the role of miRNAs in mediating these anti-mitogenic actions remains unclear. Hence, under this specific aim, we screened for E2-regulated miRNAs in SMCs.

### Introduction

Apart from promoting EC growth, migration and tube formation, E2 protects the vasculature by inhibiting vascular SMC proliferation and migration [124-127, 138], which contributes to vascular remodeling leading to occlusion [105]. Although miRNAs have been shown to mediate the actions of E2 in several tissues and cell lines [88], the role of miRNAs in mediating the effects of E2 on SMCs remains largely undefined. In section 5.1-5.3, we provided evidence that E2 regulates two miRNAs in HUVECs, miR-221 and miR-193a, and that the beneficial effects of E2 on ECs are potentially mediated by these miRNAs.

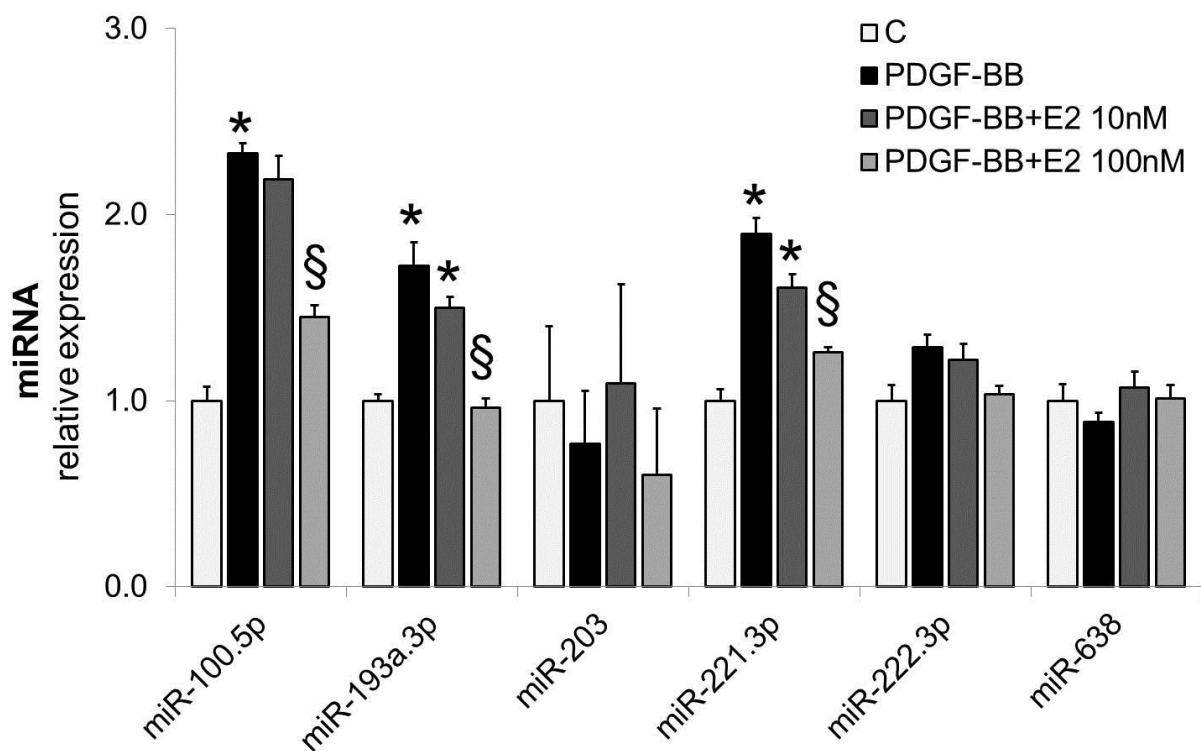
Based on our findings and the fact that miRNAs play a role in regulating SMC growth, we hypothesize that the anti-mitogenic actions of E2 on vascular SMCs are also potentially mediated through the regulation of miRNAs. Hence, under this specific aim, we screened for miRNAs that were regulated by E2 in SMCs, by searching the literature for interesting miRNAs and subsequently validating the candidates by RT-qPCR in HCASMCs.

### 5.4.1 E2 down-regulates miR-193a and miR-221 in HCASMCs

E2 protects the vascular system by abrogating the mitogenic and motogenic activities of growth factors, such as PDGF-BB. Hence, employing HCASMCs we analyzed the effect of E2 on miRNAs following stimulation with PDGF-BB. The cells were treated 24 hr before RNA extraction and miRNA analyzed by RT-qPCR. We found that expression of 3 out of 6 miRNAs that we tested was significantly induced by PDGF-BB: miR-100.5p, miR-193a.3p and miR-221.3p (from 100% to  $233\pm5\%$ ,  $172\pm12\%$ , and  $189\pm9\%$ , respectively; **Figure 39**). Importantly, the PDGF-BB-induced expression of these 3 miRNAs was abrogated in HCASMCs simultaneously treated with E2, and these effects were concentration-dependent and reached significance at

100 nM. In fact, comparing the expression of miR-100.5p, miR-193a.3p and miR-221.3p after PDGF-BB treatment alone and in the presence of 100 nM E2, the expression was reduced by  $87\pm6\%$ ,  $77\pm5\%$  and  $63\pm3\%$ , respectively ( $p<0.05$ ).

From the other tested miRNAs, our results indicate that miR-222.5p follows a similar trend in expression level, showing an increase by PDGF-BB and a concentration dependent reduction by E2 treatment (n.s.). Finally, miR-203 and miR-638 were unaffected by either PDGF-BB or E2.



**Figure 39: E2-regulated miRNAs in HCASMCs.** Cells were grown to 60% confluency in complete media prior to 24 hr treatment with or without PDGF-BB (20 ng/ml) and 0, 10 and 100 nM E2. Total RNA was extracted and relative miRNA expression levels were determined by RT-qPCR using TaqMan miRNA assays. The results were normalized to U48 and U49.  $n=3$ , \* $p<0.05$  compared to control, § $p<0.05$  compared to PDGF-BB.

### 5.4.2 Discussion

The protective effects of estrogens in the cardiovascular system are well established and include the inhibition of vascular SMC growth and migration and the prevention of injury-induced intimal hyperplasia; however, the mechanisms involved remain unclear. The role of miRNAs in the vasculature has been extensively studied [193-195]. Recently, estrogens have been associated with miRNA expression [83, 84] and

several studies have shown that E2-regulated miRNAs potentially mediate their actions in different tissues, including the vascular system [90-93]. Hence, to assess the role of miRNAs in mediating the protective effects of E2 in vascular SMCs, we searched the literature for relevant miRNAs and investigated their expression upon treatment with E2 in HCASMCs.

We have found that treatment with PDGF-BB significantly induces the expression of miR-100, miR-193a and miR-221 and that E2 inhibits PDGF-BB-induced expression of these miRNAs in a concentration dependent manner. The expression of miR-222 showed a similar trend, though the changes are not significant. The other tested miRNAs, miR-203 and miR-638 were unaffected.

From the 6 miRNAs we tested, 5 were associated with vascular SMC growth: miR-100, miR-203, miR-221, miR-222 and miR-638. Among these, miR-100, miR-203 and miR-638 exhibit anti-mitogenic effects in vascular SMC: miR-100 attenuates murine vascular SMC proliferation and migration by suppressing the mammalian target of rapamycin (mTOR) [237], miR-203 specifically inhibits mouse aortic SMC proliferation [94] and miR-638 is highly expressed in quiescent cells and abrogates PDGF-BB-induced cyclin D1 expression, cell proliferation and migration in human aortic SMCs [290]. In contrast, miR-221 and miR-222 play a prominent role in the vascular tissue by supporting de-differentiation, proliferation and migration of vascular SMCs [221-223, 230]. Association with estrogens has been demonstrated for miR-203, miR-221, miR-222 and miR-193a. The anti-mitogenic actions of E2 in vascular SMCs are mediated by miR-203 [94]. Moreover, there is evidence that E2 down-regulates miR-193a, miR-221 and miR-222 expression in MCF-7 cells [87, 92, 93, 291].

In contrast to the results from Zhao et al. [94] showing that miR-203 mediates the effects of E2 in vascular SMCs; we did not observe any change in miR-203 in HCASMCs treated with E2. Similarly, we could not confirm the findings from Li et al. [290] showing PDGF-BB dependent down-regulation of miR-638. A potential reason for the discordant findings might be the difference in cell type and the species of origin. Indeed, miR-638 was studied in human aortic SMC, whereas miR-203 was analyzed in murine cells.

The miR-222 gene is part of a gene cluster with the miR-221 gene on the X chromosome, and the expression of these miRNAs seems to be co-regulated in

some tissues as they share similar nucleotide sequences and are transcribed together as a single long non-coding RNA precursor [221, 222]. However, in support of our findings, there is evidence for differential regulation of miR-221 and miR-222. For instance, Davis et al. [223] showed that miR-221 is strongly induced by PDGF-BB treatment in PSMCs, while expression of miR-222 was rather decreased. It is therefore feasible that correlation of miR-221 and miR-222 expression depends on particular cellular/ experimental conditions, cell type and stimuli.

Among the regulated miRNAs, we found that PDGF-BB up-regulated miR-100, suggesting a positive role for this miRNA in proliferation processes. This is in contrast to the findings of Grundmann et al. [237], who demonstrated reduced vascular SMCs proliferation after transfection of human aortic SMC with pre-miR-100.

In the studies described in the previous chapters we showed that E2 down-regulates miR-193a and miR-221 in HUVECs. Moreover, we discovered that miR-193a inhibits HUVEC proliferation, migration and vasculogenesis, similar to miR-221, and that both miRNAs potentially mediate the actions of E2 in ECs. Several studies report, that the effects of the cell specific miR-221 on proliferation and migration are opposite between ECs and vascular SMCs, inasmuch that it inhibits EC functions while it stimulates vascular SMCs. Based on our observations that E2 down-regulates PDGF-BB-induced miR-221 in HCASMCs, it is feasible that E2 mediates its anti-mitogenic action on vascular SMCs via inhibition/ down-regulation of the proliferation- and migration-inducing miR-221. Although the role of miR-193a in vascular cells has not yet been elucidated, we provide evidence for inhibition of HUVEC function. There is also evidence for an anti-mitogenic role for miR-193a in different cancer cells [238-245]; however, we found that miR-193a is up-regulated by PDGF-BB, suggesting that it might be involved in the promotion of cell growth. In agreement with this hypothesis, Wang et al. supports a pro-proliferative role for miR-193a in bone mesenchymal stem cells [292], indicating that it may play contrasting dual functions depending on the tissue or cell type where it is expressed. Therefore, it is feasible that, similar to miR-221, miR-193a positively contributes to vascular SMC proliferation and migration and exhibits differential effects in vascular cells.

In summary, we discovered that the expression of miR-100, miR-193a and miR-221 is induced by PDGF-BB, suggesting a growth promoting role in vascular SMC. Moreover, these miRNAs are down-regulated by E2, indicating that they could be

potential mediators of the inhibitory actions of E2 in HCASMCs. For in-depth assessment we further investigated the impact of miR-193a and miR-221 on HCASMCs, since miR-100 was not regulated by E2 in HUVECs.



## 5.5 The role of miR-193a and miR-221 in SMCs

### Objective

Our findings revealed that E2 down-regulates miR-193a and miR-221 in HCASMCs. Since E2-dependent vaso-protection is largely due to inhibition of SMC proliferation and migration, we investigated the role of miR-193a and miR-221 in these processes. Moreover, we examined whether these miRNAs mediate the beneficial actions of E2.

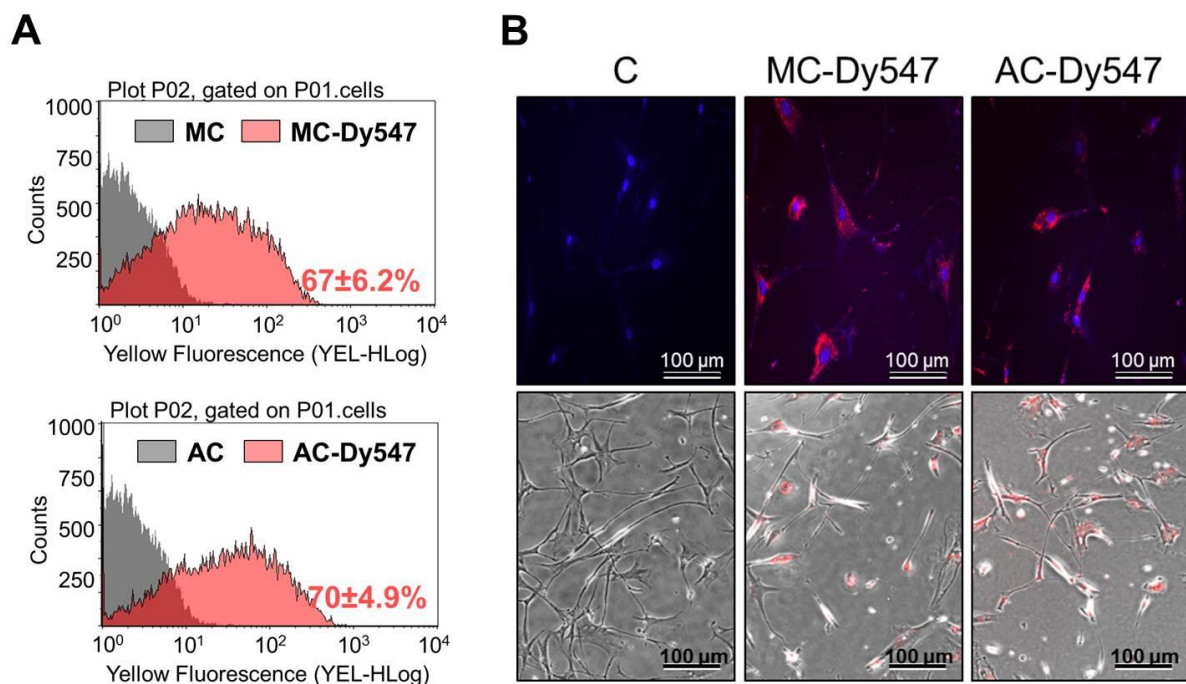
### Introduction

The function of miR-221 in vascular SMCs has been elucidated by several studies. It stimulates proliferation and migration and inhibits apoptosis [230]. Moreover, knock-down of miR-221 inhibits intimal thickening after vascular injury [222]. Compared to miR-221, the impact of miR-193a on vascular SMCs remains unknown. In our experiments we observed a significant increase in miR-193a expression in HCASMCs stimulated with PDGF-BB, suggesting that it may be positively correlated with vascular SMC proliferation. Although we found that miR-193a inhibits EC growth and function (section 5.2), Wang et al. demonstrated that miR-193a induces proliferation in bone mesenchymal stem cells [292], indicating that miR-193a may have cell specific effects. Indeed, differential effects of miRNAs on growth of vascular SMCs and ECs are well documented.

Based on the above findings, we hypothesize that miR-193a may have similar growth modulatory effects as miR-221 in vascular SMCs. Moreover, we postulate that the effects of miR-193a and miR-221 are opposite to the anti-mitogenic actions of E2. Furthermore, we hypothesize that the inhibitory effects of E2 on vascular SMCs are, at least in part, due to the down-regulation of miR-193a and miR-221. To explore this hypothesis, we first confirmed the role of miR-221 and examined the effects of miR-193a on HCASMC proliferation and migration using synthetic Mimics and Antimirs to specifically modulate the expression of miRNAs. Second, we compared the effects of miRNA inhibition with the action of E2. Finally we investigated the role of the two miRNAs in mediating the beneficial effects of E2 by restoring the expression of miR-193a and miR-221 after treatment with E2.

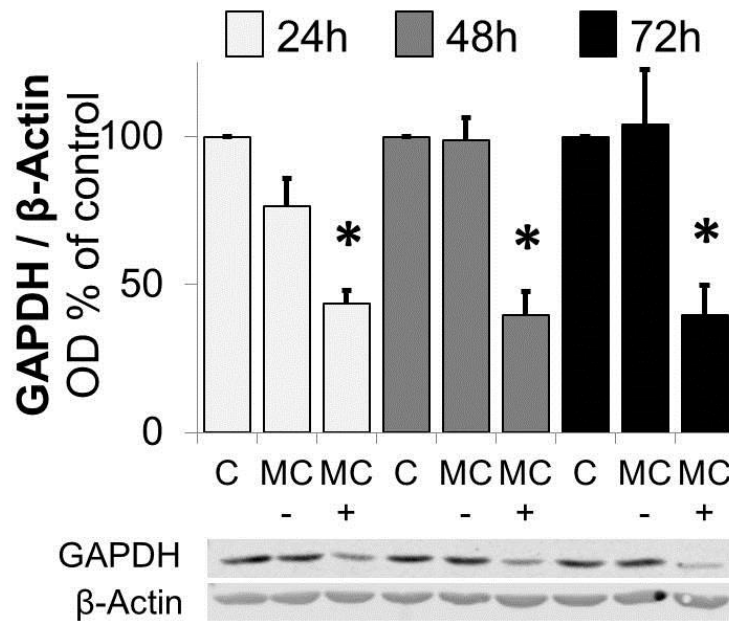
### 5.5.1 Mimics and Antimirs dysregulate the expression of miRNAs

To determine the biological effects of miR-193a and miR-221 in HCASMCs, the levels of these miRNAs were increased by transfection of the cells with miRNA Mimics, while down-regulation was achieved using the respective miRNA Antimirs. Similar to HUVECs, the transfection success of HCASMCs was assessed using the fluorescent Dy547-labelled controls and quantified by flow cytometry. Transfection efficiency was  $67\pm6.2\%$  for the Dy547-labelled Control Mimic and  $70\pm4.9\%$  for the Dy547-labelled Control Antimir (**Figure 40A**). Representative images were taken with a fluorescence microscope to visualize the transfection efficiency (**Figure 40B**).



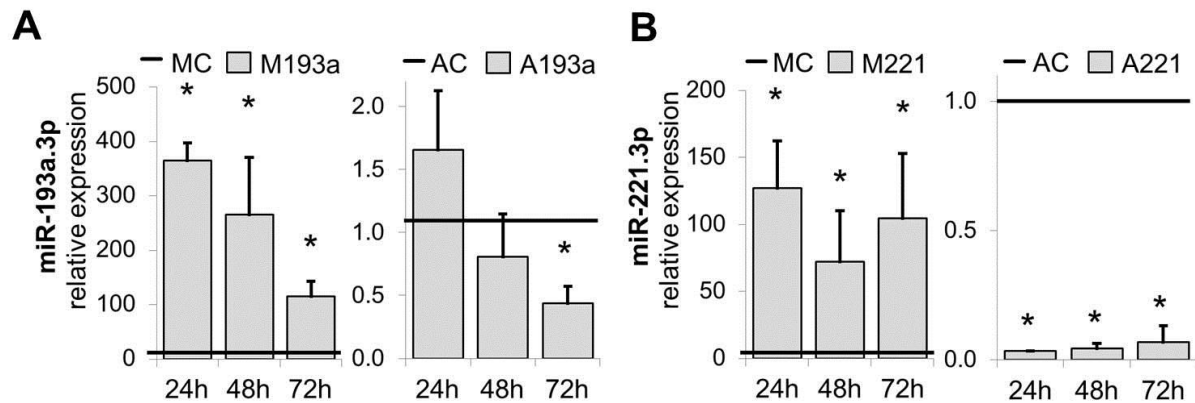
**Figure 40: Transfection efficiency of miRNA Mimic and Antimir.** Cells were transfected with 25 nM of Dy547-labelled Control Mimic (MC-Dy547) or 25 nM of Dy547-labelled Control Antimir (AC-Dy547) using Lipofectamine 2000 for 6 hr in the absence of antibiotics and serum. **Panel A:** The percentage of Dy547-positive cells was measured using flow cytometry. **Panel B:** Representative images were taken using bright field and fluorescence microscopy (Red = Dy547-labeled Mimic or Antimir; blue = Hoechst33342 stain). n=3.

Moreover, we confirmed that the experimental set up for the transfection is working optimally by means of a positive Control Mimic specifically targeting GAPDH. Protein expression of GAPDH was significantly inhibited at each tested time point post-transfection by  $43\pm5\%$  after 24 hr,  $60\pm8\%$  after 48 hr and  $62\pm10\%$  after 72 hr (**Figure 41**).



**Figure 41: Confirmation of optimal Mimic transfection conditions.** HCASMCs transfected with 25 nM GAPDH-targeting Positive Control Mimic (MC+), Negative Control (MC-) or mock transfected (C) were cultured in growing media for 24 hr, 48 hr and 72 hr prior to cell lysis and analysis of GAPDH protein expression by Western Blot.  $\beta$ -Actin was used as a loading control.  $n=3$ , \* $p<0.05$  compared to MC-.

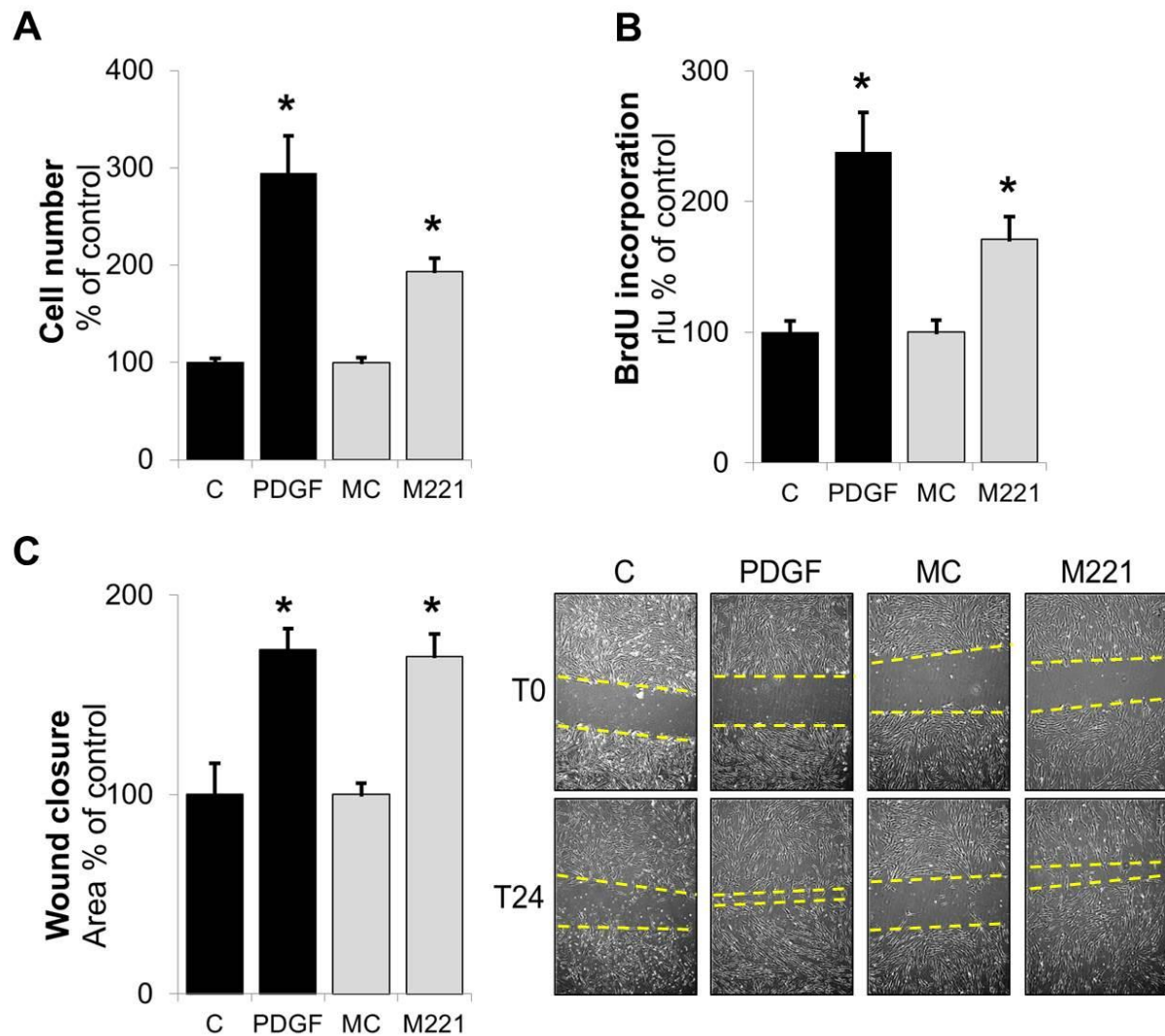
In order to confirm the alterations in miRNA levels by the Mimics and Antimirs, we isolated RNA from transfected cells and determined miR-193a as well as miR-221 levels by RT-qPCR (**Figure 42A and 42B**). Both miR-193a and miR-221 Mimics (M193a and M221, respectively) substantially up-regulated the respective miRNA levels after 24 hr, 48 hr and 72 hr. Transfection with the Antimir for miR-193a reduced miR-193a levels after 48 hr (by  $20\pm34\%$ , n.s.) and 72 hr (by  $56\pm14\%$ ,  $p<0.05$ ), whereas miR-221 level was significantly inhibited by  $\sim 90\%$  at each tested time point by its Antimir.



**Figure 42: Transfection with Mimics and Antimirs changes the levels of the respective miRNAs.** HCASMCs were transfected either with 25 nM miR-193a Mimic or Antimир (M193a, A193a; **Panel A**) or 25 nM miR-221 Mimic or Antimир (M221, A221; **Panel B**) or the respective negative Controls (MC, Mimic Control; AC, Antimир Control). The cells were kept in growing media 24 hr, 48 hr and 72hr post-transfection before RNA was extracted and the miRNA level determined by RT-qPCR. n=3, \*p<0.05 compared to the respective control.

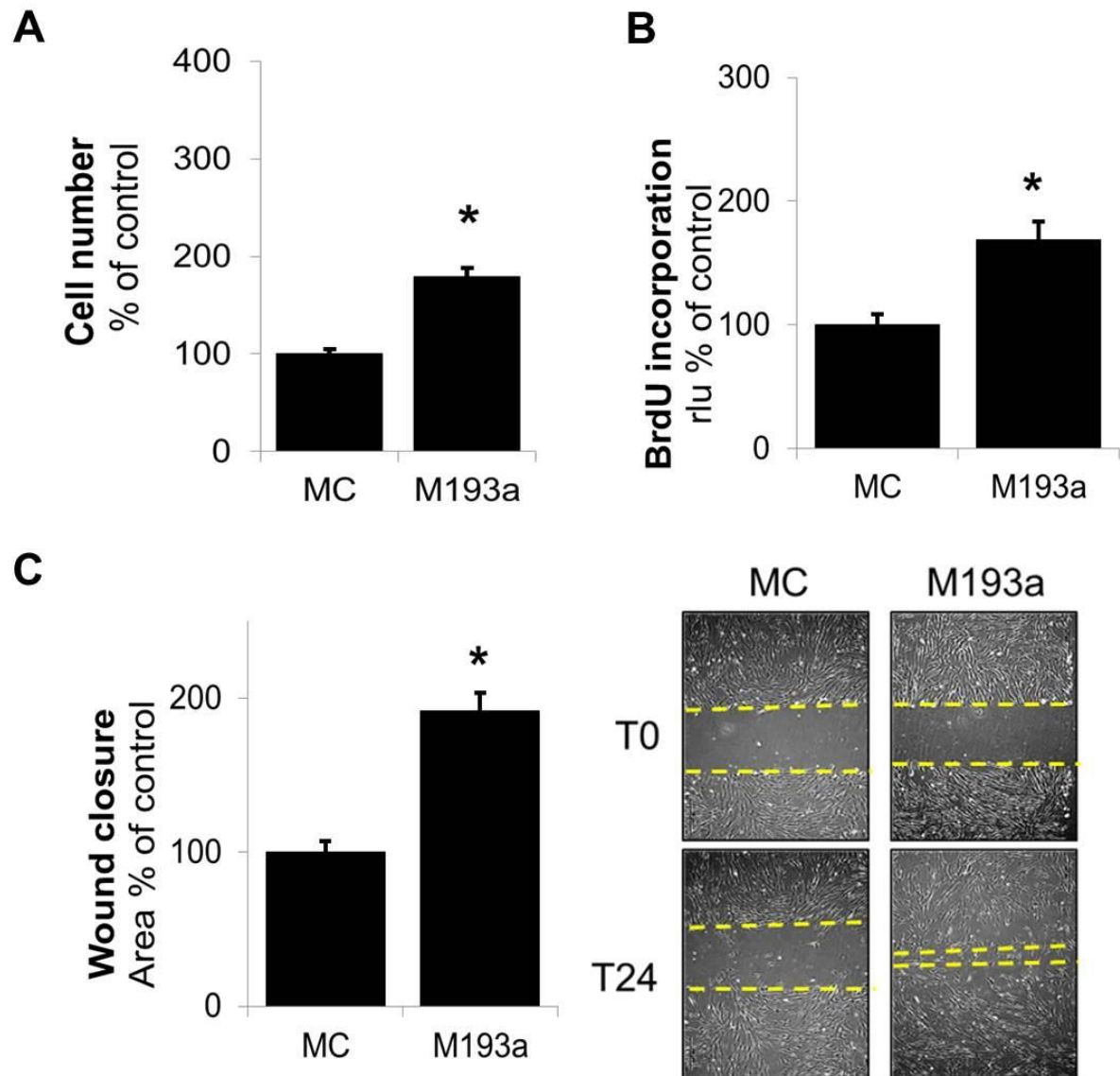
### 5.5.2 miR-193a and miR-221 induce HCASMC proliferation and migration

We first confirmed the growth promoting role of miR-221 in vascular SMCs and compared it with the effects of PDGF-BB. Similar to PDGF-BB, transfection of HCASMCs with miR-221 Mimic induced proliferation, as assessed by cell counting and analysis of DNA synthesis using BrdU incorporation assay. Both PDGF-BB and M221 induced cell number by  $194 \pm 39\%$  and  $93 \pm 13\%$ , respectively ( $p < 0.05$ ; **Figure 43A**). DNA synthesis was significantly increased from  $100 \pm 8\%$  to  $237 \pm 30\%$  by PDGF-BB and from  $100 \pm 4\%$  to  $171 \pm 17\%$  by miR-221 Mimic (**Figure 43B**). Moreover, we investigated the role of miR-221 on HCASMC migration by means of a scratch wound assay. Both PDGF-BB and M221 accelerated wound closure (**Figure 43C**) by  $72 \pm 10\%$  and  $69 \pm 11\%$ , respectively, ( $p < 0.05$ ) thus confirming the stimulatory role of miR-221.



**Figure 43: miR-221 overexpression induces HCASM C proliferation and migration similar to PDGF-BB.** HCASMCs were either treated with or without PDGF-BB (PDGF, 20 ng/ml) or transfected with miR-221 Mimic (M221) or Control Mimic (MC). All the assays were conducted in serum free media. **Panel A:** Cell number was assessed 3 days after transfection. **Panel B:** BrdU incorporation was determined using a BrdU ELISA kit. **Panel C:** Cell migration was determined using a wound closure assay. The cells were grown in complete medium for 48 hr after transfection and prior to making the wound. Representative images for each condition were taken at time 0 (T0) and 24 hr later (T24) as indicated.  $n=3$ , \* $p<0.05$  compared to the respective control (C for PDGF-BB, MC for M221).

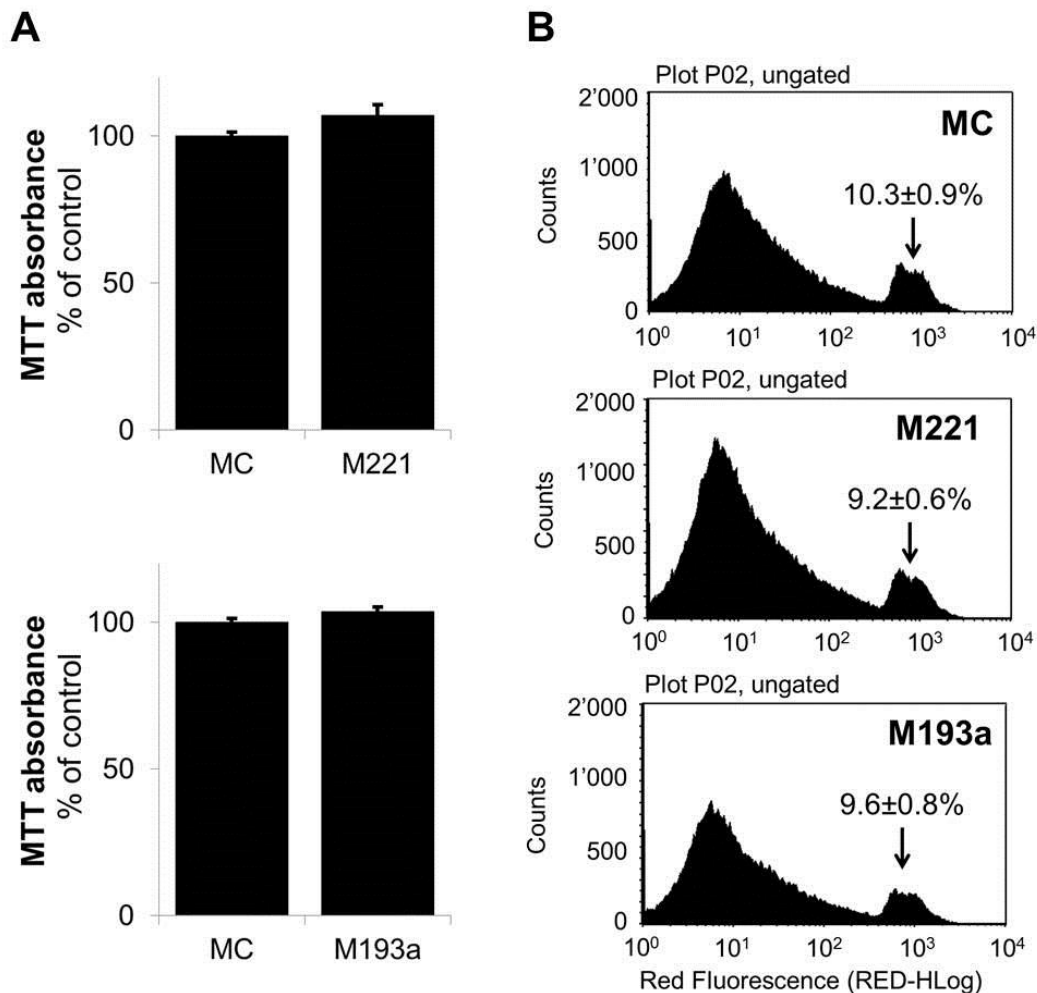
In parallel experiments we studied the effect of miR-193a up-regulation on both proliferation and migration of HCASMCs. Analogous to M221, miR-193a Mimic M193a induced cell number and DNA synthesis (by  $79. \pm 8\%$  and  $68 \pm 15\%$ , respectively,  $p<0.05$ ; **Figure 44A and 44B**). Similarly, transfection of the cells with M193a significantly accelerated wound closure by  $91 \pm 12\%$  (**Figure 44C**).



**Figure 44: miR-193a overexpression induces HCASMC proliferation and migration.** Cells were transfected with miR-193a Mimic (M193a) or Control Mimic (MC). All the assays were made in serum free media. **Panel A:** Cell number was assessed 3 days after transfection. **Panel B:** BrdU incorporation was determined using a BrdU ELISA kit. **Panel C:** Cell migration was determined using a wound closure assay. Cells were grown in complete media for 48 hr after transfection, before the wound was made. Representative images for each condition at time 0 (T0) and 24 hr later (T24) are shown.  $n=3$ ,  $*p<0.05$  compared to control.

The effects of the two miRNAs on proliferation and migration might be due to changes in cell viability. To rule out this possibility we investigated whether overexpression of miR-221 or miR-193a affects cell viability. As shown in **Figure 45**, neither of the two miRNAs had an effect on cell viability, as assessed by MTT test

and PI staining followed by flow cytometric analysis. Compared to the MC, M221 and M193a increased MTT absorbance only by  $7\pm4\%$  and  $3\pm2\%$ , respectively (n.s.; **Figure 45A**). These results were also confirmed by the PI staining, which showed no significant change in Control and Mimic treated cells. The MC displayed  $10.3\pm0.9\%$ , M221 had  $9.2\pm0.6\%$  and M193a showed  $9.6\pm0.8\%$  PI positives (**Figure 45B**).



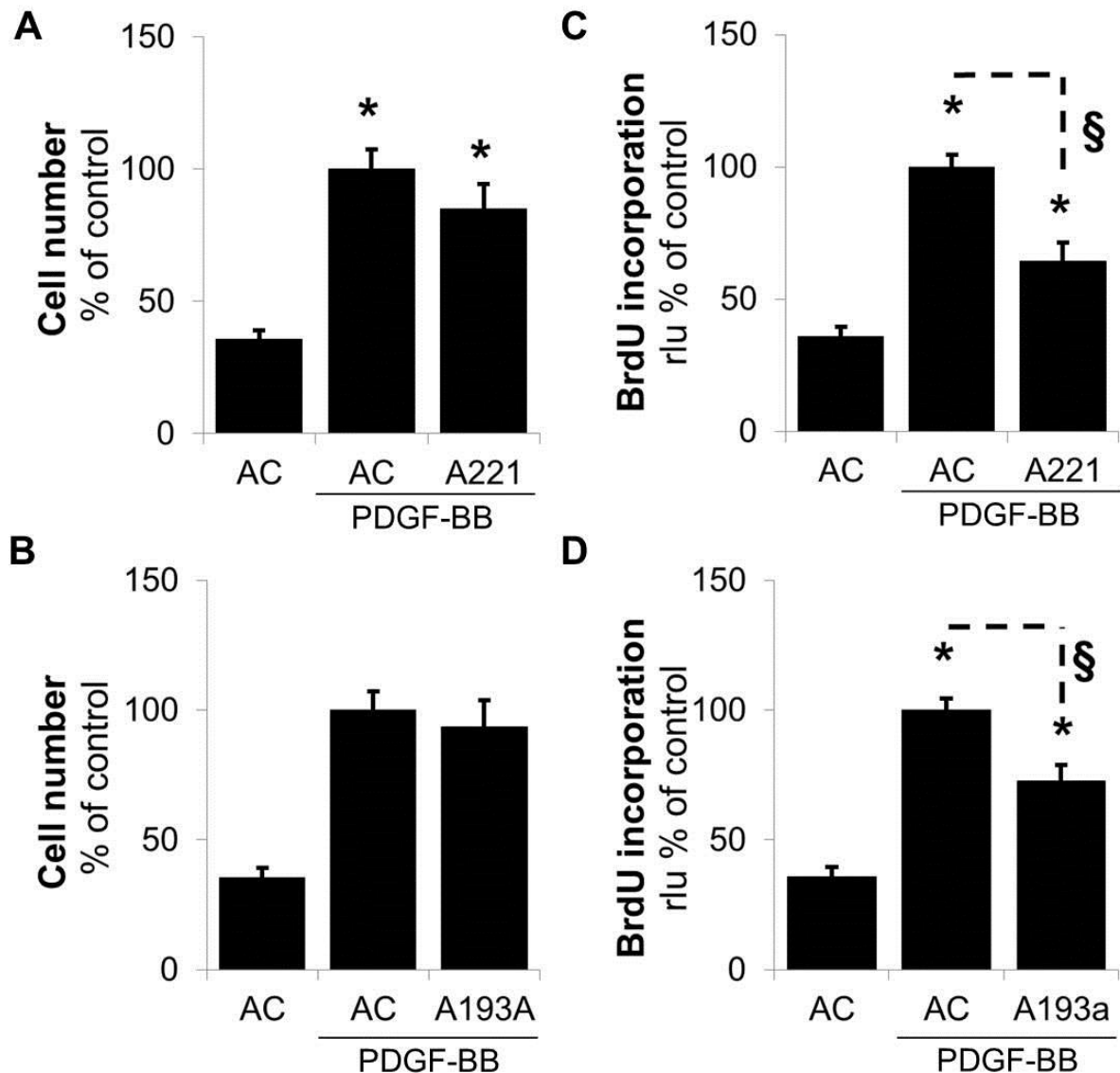
**Figure 45: miR-221 and miR-193a overexpression does not affect cell viability.** HCASMCs were transfected with miR-221 Mimic (M221), miR-193a Mimic (M193a) or the Control Mimic (MC) and kept in growing media 24 hr after transfection. **Panel A:** For the MTT assay the cells were incubated 1 hr with 0.5 mg/ml MTT before measuring the absorbance was measured. **Panel B:** For the PI staining cells were trypsinized, incubated for 5 minutes with 0.2  $\mu\text{g/ml}$  PI and analyzed with a flow cytometer. The percentage of dead cells is indicated.  $n=3$ ,  $*p<0.05$  compared to the respective control.

### 5.5.3 miR-193a and miR-221 Antimirs mimic the effects of E2

In contrast to the Mimics, we expected down-regulation of miR-193a and miR-221 by the Antimirs to have an inhibitory action on PDGF-BB-induced HCASMC proliferation.



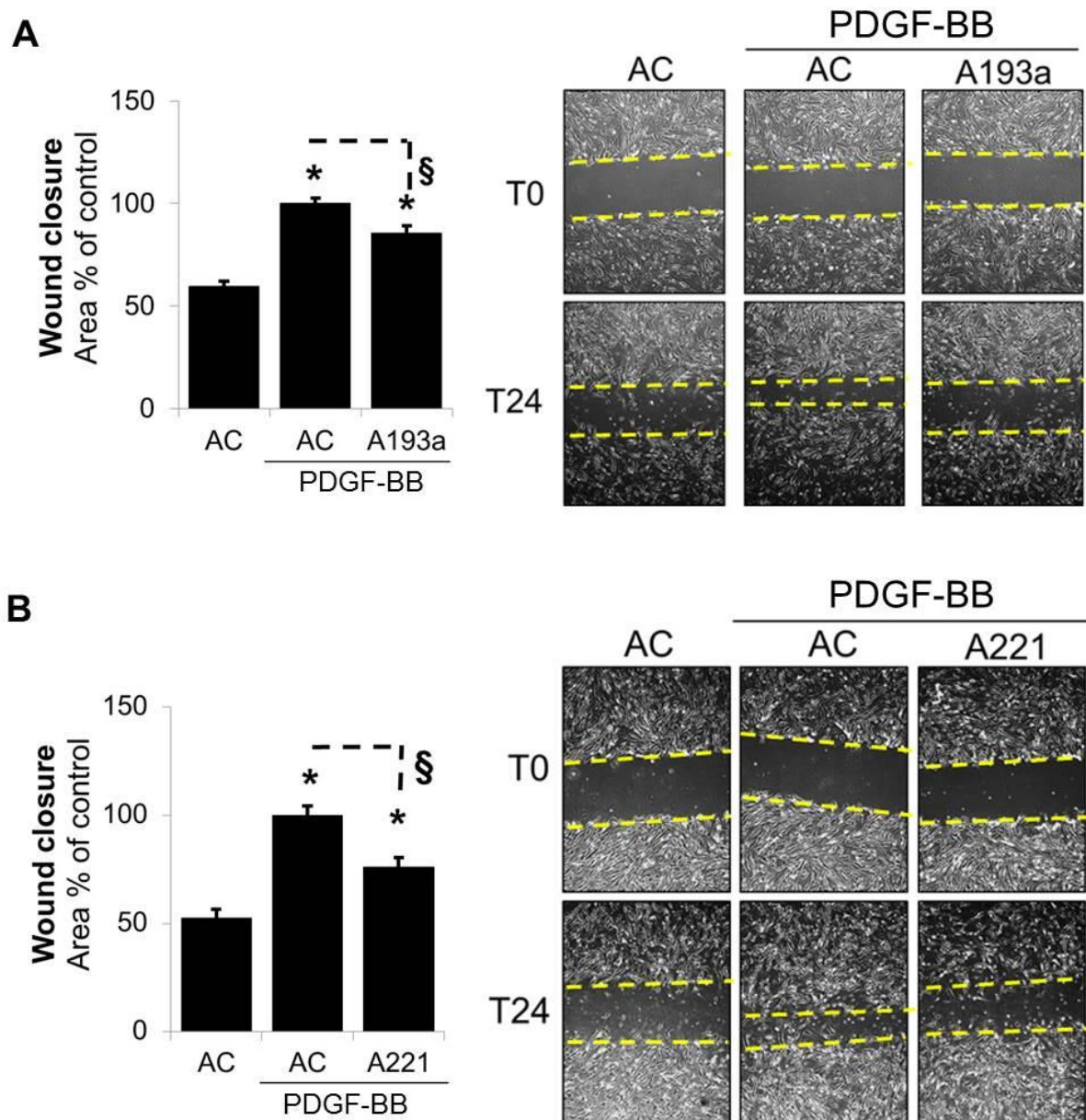
Unfortunately, A193a and A221 only slightly reduced cell number by  $7\pm 10\%$  and  $15\pm 9\%$ , respectively (**Figure 46A and 46B**). However, the more sensitive BrdU incorporation assay showed that both A193a and A221 are able to inhibit BrdU incorporation from  $100\pm 5\%$  to  $72\pm 6\%$  and  $64\pm 7\%$ , respectively ( $p<0.05$ ; **Figure 46C and 46D**).



**Figure 46: miR-221 and miR-193a down-regulation inhibits HCASMC proliferation.** Cells were transfected with miR-221 Antimир (A221), miR-193a Antimир (A193a) or Antimир Control (AC) prior to treatment with 20 ng/ml PDGF-BB. **Panel A and B:** Cell number was assessed by counting of the cells 3 days after transfection. **Panel C and D:** DNA synthesis was measured using a BrdU incorporation ELISA kit.  $n=3$ , \* $p<0.05$  compared to control, § $p<0.05$  as indicated.

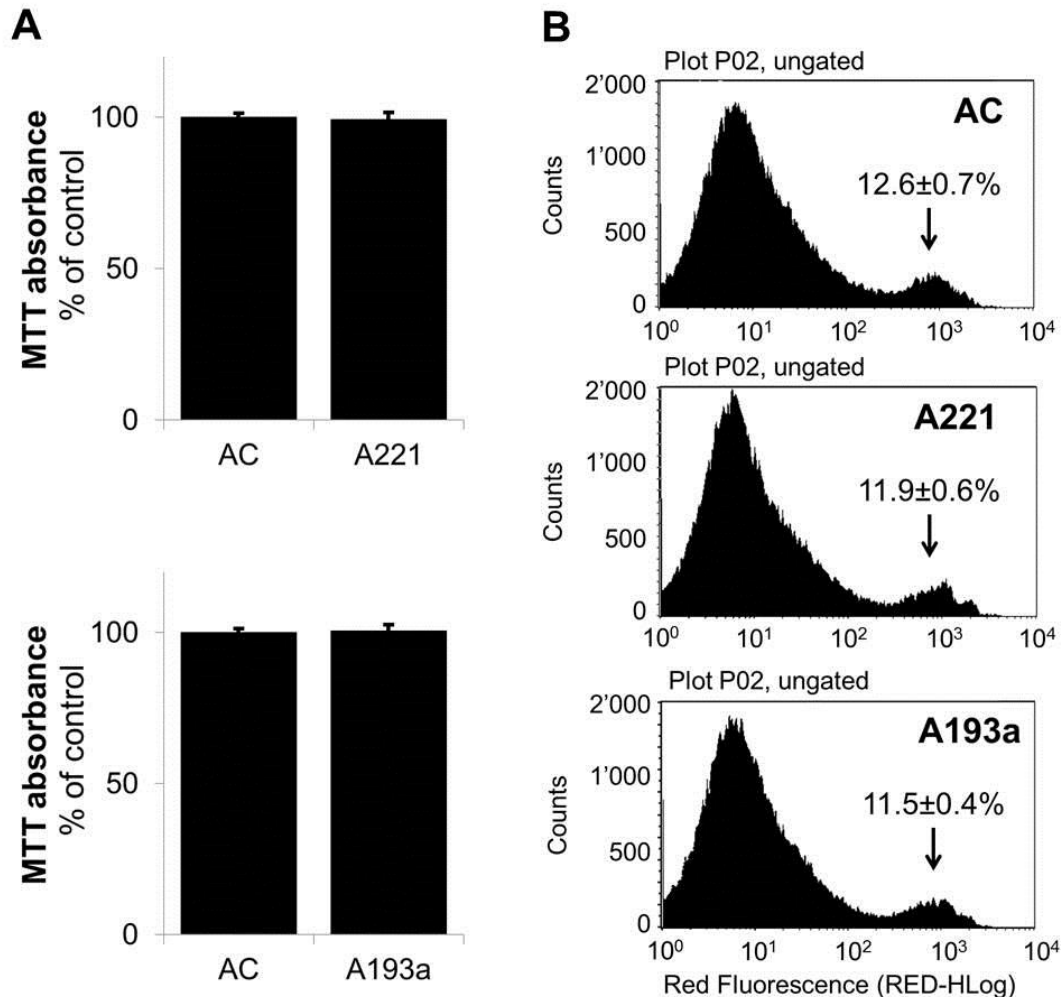


Moreover, down-regulation of miR-193a and miR-221 significantly inhibited PDGF-BB-induced wound closure. The Antimir A193a reduced the scratch induced wound by  $15\pm3\%$ , while A221 showed a stronger effect on cell migration by reducing it from  $100\pm4\%$  to  $76\pm4\%$  (**Figure 47A and 47B**).



**Figure 47: miR-221 and miR-193a down-regulation inhibits HCASMC migration.** Cells were transfected with miR-193a Antimir (A193a; **Panel A**), miR-221 (A221; **Panel B**) or Antimir Control (AC) prior to treatment with 20 ng/ml PDGF-BB. Migration was determined using a wound closure scratch assay. Cells were grown in complete media for 48 hr after transfection and before making the wound. Photomicrographs depict representative images of each condition after scratch at time 0 (T0) and 24 hr later.  $n=3$ ,  $*p<0.05$  compared to control,  $\$p<0.05$  as indicated.

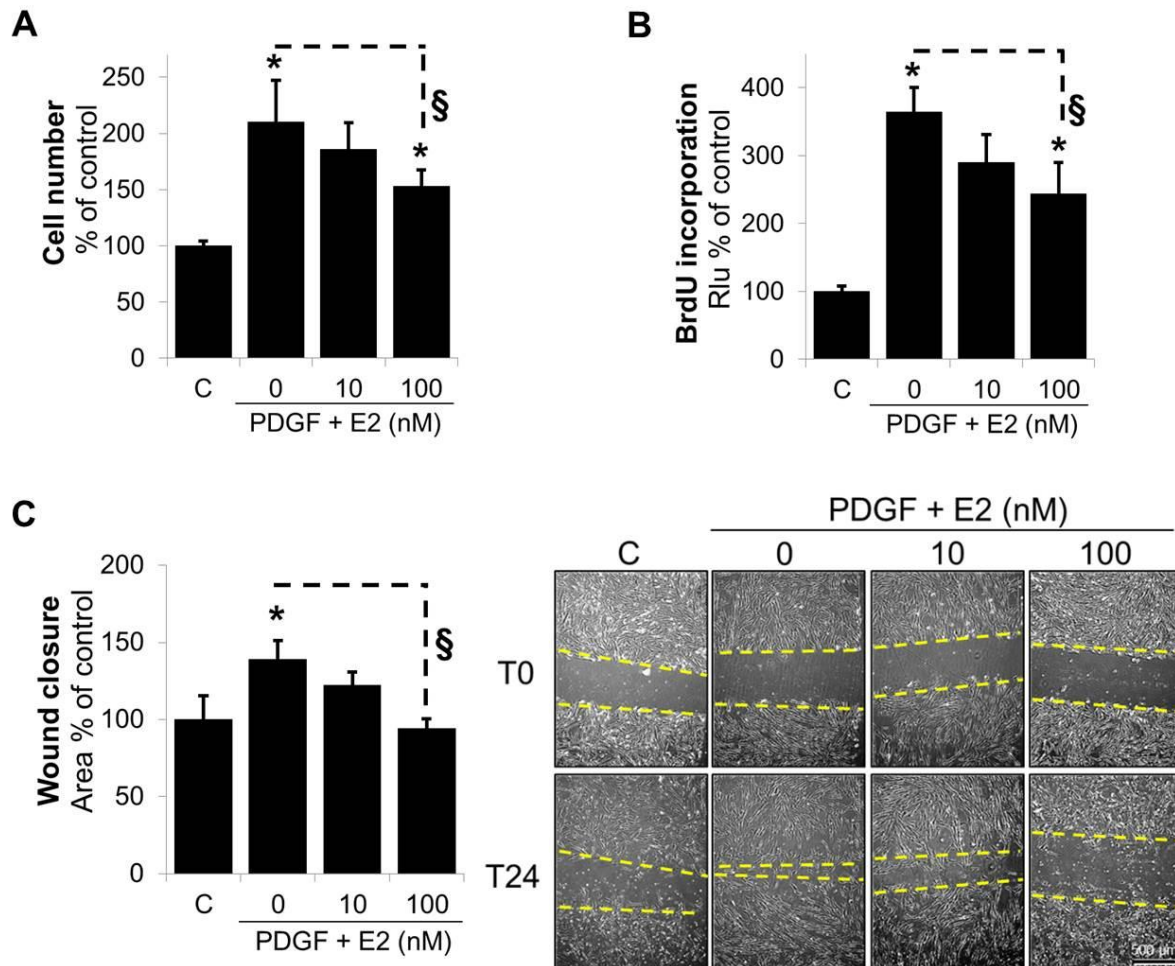
As for the Mimics, we assessed the role of the Antimirs on cell viability by MTT test and PI staining followed by flow cytometric analysis (**Figure 48A and 48B**). We found that silencing of both miRNAs neither led to changes in MTT absorbance nor in the profile of the PI-stained cells, ( $12.6\pm0.7\%$ ,  $11.9\pm0.6\%$  and  $11.5\pm0.4\%$ ).



**Figure 48: miR-221 and miR-193a down-regulation does not affect cell viability.** HCASMCs were transfected with miR-221 Antimir (A221), miR-193a Antimir (A193a) or the Control Antimir (AC) and kept in growing media for 24 hr after transfection. **Panel A:** For the MTT assay the cells were incubated for 1 hr with 0.5 mg/ml MTT before measuring the absorbance. **Panel B:** For the PI staining the cells were trypsinized, incubated 5 minutes with 0.2  $\mu$ g/ml PI and analyzed with a flow cytometer. The percentage of dead cells is indicated.  $n=3$ ,  $*p<0.05$  compared to control.

The effects of the two Antimirs on HCASMC growth were similar to that of E2. In fact, E2 inhibited PDGF-BB-induced proliferation concentration dependently, as assessed by cell counting (**Figure 49A**) and BrdU incorporation (**Figure 49B**). At a concentration of 100 nM E2 significantly inhibited cell number and DNA synthesis (by

30±44% and 33±11%, respectively). Furthermore the effects of E2 on HCASMC migration were comparable to those following miR-193a and miR-221 down-regulation. E2 reduced the PDGF-BB-induced wound closure concentration dependently and 100 nM E2 significantly decreased it from 100±5% to 73±4% (Figure 48C).

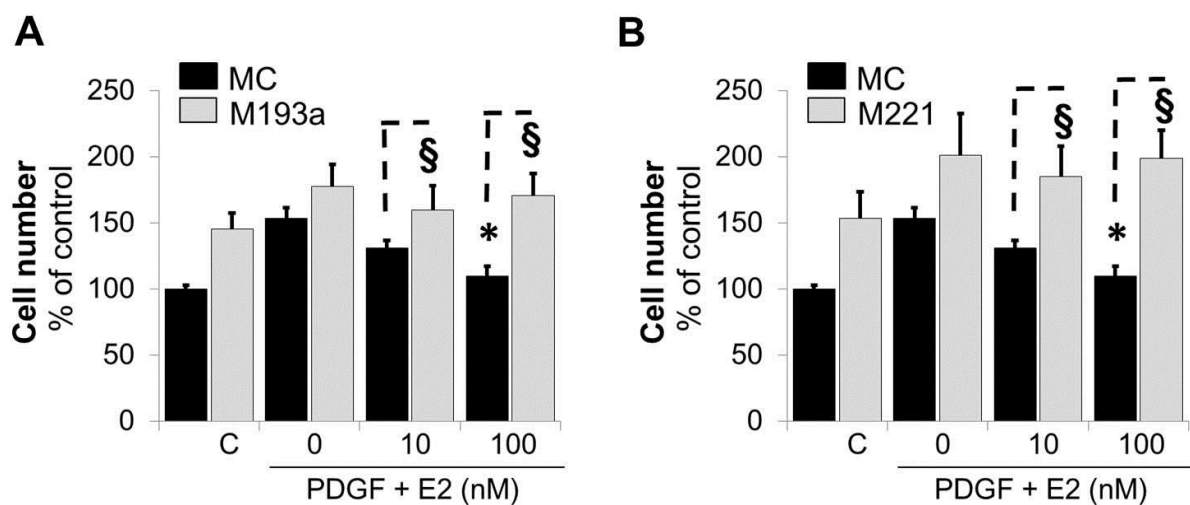


**Figure 49: E2 inhibits HCASMC proliferation and migration.** Cells were treated with 20 ng/ml PDGF-BB (PDGF) and 0, 10 and 100 nM E2. All the assays were made in serum free media. **Panel A:** Cell number was assessed 3 days after transfection. **Panel B:** BrdU incorporation was determined using a BrdU ELISA kit. **Panel C:** Cell migration was determined using a wound closure assay. The cells were grown in complete media for 48 hr after transfection, before the wound was made. Representative images for each condition at time 0 (T0) and 24 hr later (T24) are shown. n=3, \*p<0.05 compared to control, §p<0.05 as indicated.

#### 5.5.4 miR-193a and miR-221 abrogate the protective actions of E2

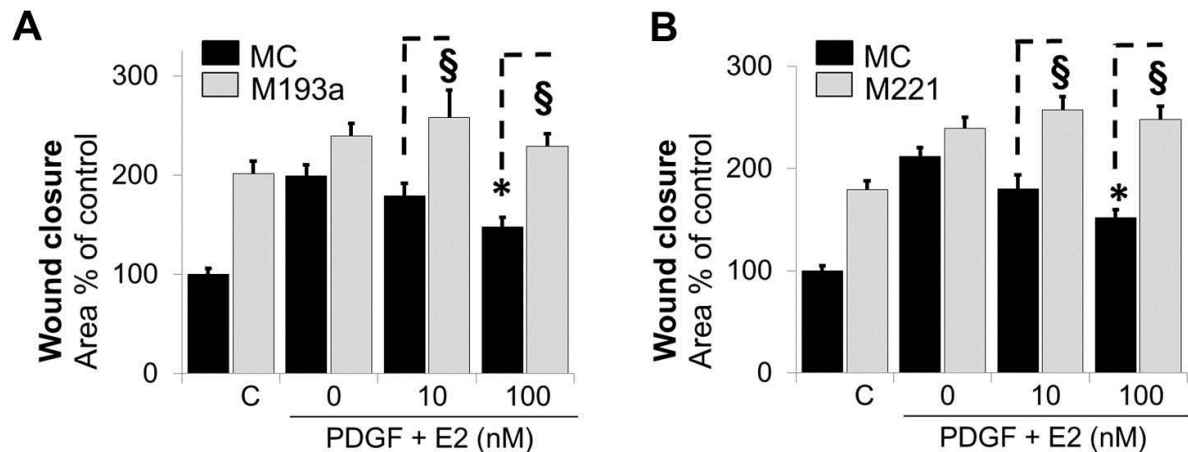
Since we previously found that E2 inhibits miR-193a and miR-221 expression in HCASMCs and that down-regulation of both miRNAs had comparable effects to E2

on HCASMCs growth, we hypothesized that the protective effects of E2 are, at least in part, due to its modulation of miR-193a and miR-221. In order to verify this hypothesis, overexpression of miR-193a and miR-221 was combined with E2 treatment. Indeed, transfection of the cells with either M193a or M221 significantly reversed the inhibitory effects of E2 on cell proliferation at both 10 and 100 nM. M193a increased cell number from  $131\pm6\%$  to  $159\pm18\%$  (10 nM E2) and from  $110\pm7\%$  to  $170\pm17\%$  (100 nM E2; **Figure 50A**), while M221 increased the counts from  $131\pm6\%$  to  $185\pm23$  (10 nM E2) and from  $110\pm7\%$  to  $199\pm20\%$  (100 nM E2; **Figure 50B**).



**Figure 50: miR-193a and miR-221 overexpression reverses the inhibitory effects of E2 on HCASMC proliferation.** Cells were transfected with the Control Mimic (MC), miR-193a Mimic (M193a; **Panel A**) or miR-221 Mimic (M221; **Panel B**) and immediately treated with or without E2 (10-100 nM) and 20 ng/ml PDGF-BB (PDGF). Proliferation was determined by counting the cells 3 days after transfection /treatment.  $n=3$ ,  $*p<0.05$  compared to control,  $\$p<0.05$  as indicated.

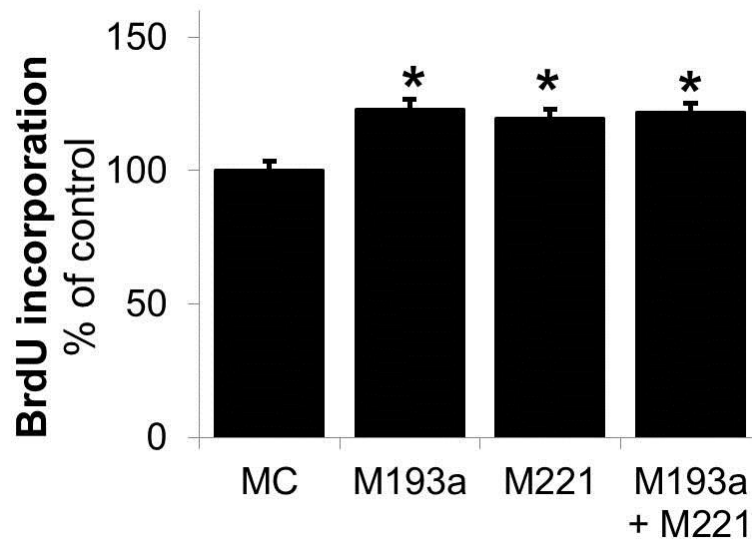
Furthermore, the wound closure scratch assay revealed similar results with regard to HCASMC migration. The inhibitory effects of 10 and 100 nM of E2 on PDGF-BB-induced migration was reversed by M193a from  $179\pm13\%$  to  $258\pm27\%$  and from  $148\pm9\%$  to  $229\pm13\%$ , respectively ( $p<0.05$ ; **Figure 51A**). Likewise, M221 significantly opposed E2 action at both 10 and 100 nM, from  $180\pm13\%$  to  $257\pm13\%$  and from  $152\pm8\%$  to  $248\pm13\%$ , respectively (**Figure 51B**).



**Figure 51: miR-193a and miR-221 overexpression reverses the inhibitory effects of E2 on HCASMC migration.** Cell migration was determined using a wound closure scratch assay. The cells were transfected with the Control Mimic (MC), miR-193a Mimic (M193a; **Panel A**) or miR-221 Mimic (M221; **Panel B**). Treatment with or without E2 (10-100 nM) and 20 ng/ml PDGF-BB (PDGF) was added after the scratch was made. Photomicrographs were taken immediately after the scratch / treatment at time 0 (T0) and 24 hr later (T24). n=3, \*p<0.05 compared to control, §p<0.05 as indicated.

### 5.5.5 miR-193a and miR-221 do not have additive effects

Since both miRNAs have a similar role in inducing HCASMC growth, we examined the combined effect of the two miRNAs on DNA synthesis, using BrdU incorporation assay (**Figure 52**). Interestingly, we found that the combination of the Mimics significantly induced BrdU incorporation from  $100 \pm 3\%$  to  $122 \pm 3\%$ . However, combined treatment did not further increase the effects compared to the individual Mimics M193a and M221, which showed an induction of  $23 \pm 4\%$  and  $20 \pm 3\%$ , respectively (p<0.05). Hence our results demonstrate no additive effect of miR-193a and miR-221 on HCASMCs growth.



**Figure 52: Combined treatment with miR-193a and miR-221 has no additive effect.** HCASMCs were transfected with 25 nM Control Mimic (MC), miR-221 Mimic (M221) or miR-193a Mimic (M193a) alone or with a combination of the two Mimics (12.5 nM each). BrdU incorporation was determined using a BrdU ELISA kit.  $n=3$ ,  $*p<0.05$  compared to control.

### 5.5.6 Discussion

The first aim of this part of the study was to confirm the proliferation and migration inducing effects of miR-221 in HCASMCs and investigate the effect of miR-193a on HCASMC function. Since PDGF-BB stimulates miR-193a and miR-221 expression in HCASMCs, we speculated that miR-193a may have stimulatory effects on HCASMCs growth similar to miR-221. Since these actions are opposite to the anti-mitogenic actions of E2, we hypothesized that down-regulation of miR-193a and miR-221 by E2 mediates its inhibitory effects on vascular SMC proliferation and migration.

Indeed, similar to PDGF-BB, miR-221 induced HCASMCs proliferation and migration, as assessed by the increase in cell number and DNA synthesis and acceleration of wound closure. Similar to PDGF-BB and miR-221 overexpression, the up-regulation of miR-193a also induced HCASMC proliferation and migration. The stimulatory role of miR-193a and miR-221 in vascular SMCs is further confirmed by our observation that neutralization of miRNA with Antimirs inhibited DNA synthesis and wound closure. Moreover, down-regulation of both miRNAs inhibited HCASMCs function comparable to treatment with E2. Finally, we demonstrate that overexpression of miR-193a or miR-221 abrogated E2-mediated inhibition of HCASMC proliferation and

migration, suggesting that down-regulation of these miRNAs contributes to the protective effects of E2 in vascular SMCs.

In order to study the physiological role of the miRNAs, we transfected the cells with miRNA Mimics or Antimirs using lipofection. The transfection efficiency was relatively good (70%), as confirmed using fluorescently labeled controls. Transfection success was further verified using a GAPDH-targeting Positive Control miRNA, which inhibited GAPDH significantly at each tested time point, suggesting that the Mimics are potentially incorporated into the RISC complex and functionally active. Moreover, the uptake of miR-193a and miR-221 Mimics and Antimirs was confirmed by RT-qPCR. The levels of both miRNAs were greatly induced after transfection with the respective Mimics. After transfection with the Antimirs, miR-221 levels were completely inhibited throughout the tested time points, while miR-193a levels were significantly reduced only after 72 hr. It is feasible that the lack of down-regulation of miR-193a is due to inhibition of miRNA activity even in absence of its degradation, as reported earlier [252, 253]. The use of Antimirs in the functional assays provides evidence that miRNA activity is inhibited, independently of the miRNA levels observed by RT-qPCR. However, off-target effects of the miRNAs cannot be excluded.

In the current study, we found that miR-221 and miR-193a are up-regulated by PDGF-BB and mimic its effects on inducing vascular SMC proliferation, suggesting that they might mediate the growth stimulatory actions of PDGF-BB. Indeed, Davis et al. demonstrated that miR-221 up-regulation by PDGF-BB mediates its pro-proliferative actions in pulmonary aortic SMCs through inhibition of c-Kit and p27 [223]. Here we provide the first evidence that miR-193a participates in the regulation of vascular SMC proliferation. These findings, together with the fact that miR-193a expression is stimulated by PDGF-BB and that PDGF-BB-induced DNA synthesis and wound closure are significantly inhibited by miR-193a down-regulation, suggests that miR-193a also mediates the mitogenic effects of PDGF-BB on HCASMCs. However, this assumption needs to be verified, for instance by observing whether PDGF-BB is able to further simulate vascular SMC activity in cells transfected with miR-193a Mimic.

Interestingly, we observed that the PDGF-BB-induced increase in cell number is only slightly reduced by miR-221 or miR-193a inhibition, while the BrdU incorporation

assay showed 30-40% decrease in DNA synthesis. An explanation for this differential effect might be the higher sensitivity of the BrdU assay as compared to cell counts or the short incubation time employed to observe a proper reduction in cell number. Since miR-221 or miR-193a down-regulation did not completely abrogate PDGF-BB-induced HCASMC growth and migration, it is likely that other molecules also participate in regulating PDGF-BB actions and inhibition of one miRNA is not enough to neutralize its actions.

Although changes in cell proliferation and migration are influenced by cell survival, we could not detect any changes in cell viability by miR-221 and miR-193a modulation. There is only one report about the effects of miR-221 on cell viability in vascular SMCs. Liu et al. showed that knock-down of miR-221/222 increased apoptosis, while overexpression had the opposite effect [230]. However, these results were obtained in rat aortic SMCs and under serum-free conditions, while we used HCASMCs and kept them in growing medium before measuring cell viability. Hence, differences in experimental conditions and cell type may account for the dissimilar results. The role of miR-193a on vascular SMC survival has not been investigated; nevertheless, its pro-apoptotic functions are reported in several tumors, including melanoma, hepatocellular carcinoma, acute myeloid leukemia, breast and prostate cancer [245]. Yet in this study we have observed that miR-193a modulation does not alter HUVEC viability, which supports our finding that HCASMC survival is also not regulated by miR-193a. Finally, we used indirect methods to study the effect of the miRNAs on cell viability and did not specifically measure apoptosis. Therefore, the analysis of apoptosis using a specific marker, such as Annexin V or TUNEL staining, might give a divergent answer about the role of miR-193a and miR-221 in vascular SMC survival.

Davis et al. additionally demonstrated that miR-221 is critical for the modulation of vascular SMC phenotype, as up-regulation of miR-221 abrogated the expression of SMC genes such as  *$\alpha$ -smooth muscle action (SMA)*, *smooth muscle calponin (CNN)* and *SM22 $\alpha$*  [223, 293]. These findings support the pro-proliferative role of miR-221, as only de-differentiated SMCs are able to reenter the cell cycle and proliferate. Unfortunately, we did not investigate the role of miR-193a in the regulation of SMC differentiation, though it could also be a potential mechanism of increased cell proliferation.



The protective actions of E2 on the vascular system include its inhibitory effects on vascular SMC proliferation and migration and the prevention of neointimal formation after injury [222]. Here we confirm the anti-mitogenic and anti-migratory actions of E2 on PDGF-BB-induced HCASMC growth. Moreover, we provide evidence that down-regulation of miR-221 and miR-193a mimics the effects of E2 on HCASMC stimulated by PDGF-BB. These findings suggest that miRNA down-regulation by E2 might mediate the inhibitory effects of E2 on HCASMCs. Indeed, re-expression of the miRNA after its down-regulation by E2 treatment reversed its inhibitory effects on proliferation and wound closure. Taken together, our findings demonstrate that knock-down of miR-221 and miR-193a abolishes E2-mediated inhibition of HCASMC proliferation and migration. Our findings identify that miR-221 and miR-193a actively contribute to the growth inhibitory effects of E2 in HCASMCs. These findings are further supported by the fact that the same miRNAs potentially mediate estrogenic and growth stimulatory actions in ECs, as we demonstrate in the previous chapters.

The mechanisms by which miRNAs modulate vascular SMCs may include the regulation of several intracellular pathways. To test whether miR-193a and miR-221 affect common or different pathways in stimulating vascular SMC proliferation, we tested and compared the individual and combined actions of both miRNAs on HCASMCs growth using BrdU incorporation assay. Since we did not observe any additive effects compared to overexpression of individual miRNAs, we speculate that miR-193a and miR-221 potentially target the same genes or different genes within the same pathway. In support of our hypothesis, other studies explain additive effects after miRNA combination by the fact that these miRNA target distinct pathways. For instance, Stahlhut et al. showed that let-7b and miR-34a synergize to inhibit cell proliferation and tumor growth by targeting different sets of oncogenes [294]. However, as already discussed in section 5.2.6, our results of the simultaneous transfection of miR-193a and miR-221 Mimics should be interpreted with caution, as we did not control whether both Mimics are taken up by the cells and are functionally active. First, the possibility that only one miRNA is efficiently transfected into the cells should be tested by RT-qPCR analysis. Second, it is necessary to examine concentration dependent effects of the Mimic, since we employed only 12.5 nM of each miRNA for the co-transfection compared to the 25 nM used for each miRNA alone. Third, since co-administered Mimics may compete for the binding of AGO2

[265], the functional activity of both miR-193a and miR-221 Mimics should be verified using a reporter assay or through AGO2 pull-down followed by RT-qPCR.

In conclusion, we hypothesize that down-regulation of miR-193a and miR-221 by E2 suppresses the stimulatory actions of miR-193a and miR-221 on proliferation, and migration of HCASMCs, thereby contributing to the inhibitory effect of E2 on these processes. Moreover, we speculate that miR-193a and miR-221 modulate mRNAs of genes within the same pathways, which are regulated by E2 and important for E2-mediated inhibition of SMC growth.

## 5.6 Intracellular mechanisms of E2-regulated miRNAs in SMCs

### Objective

Our results provide evidence that miR-193a and miR-221 mediate the anti-mitogenic actions of E2 in HCASMCs. Hence, we further investigated the potential underlying mechanisms. Since E2 inhibits SMC proliferation, in part by blocking cell cycle progression from G<sub>1</sub> to S phase, we assessed the role of the two miRNAs on the regulation of several early cell cycle proteins.

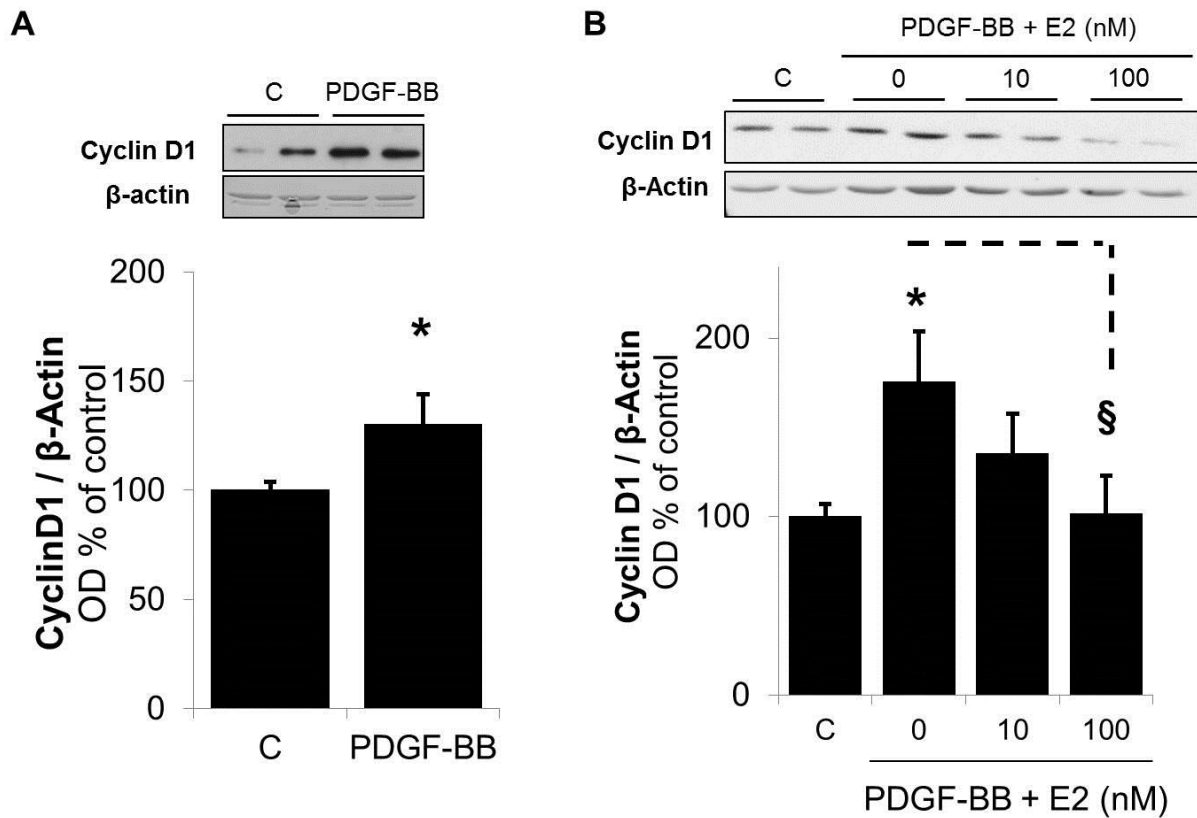
### Introduction

In section 5.5 we demonstrated that miR-193a and miR-221 mediate the inhibitory effects of E2 on proliferation and migration of vascular SMCs. Although the molecular mechanisms underlying the anti-mitogenic actions of E2 in SMCs are far from clear, current evidence indicates that E2 inhibits SMC proliferation in part by blocking cell cycle progression from G<sub>1</sub> to S phase by regulating several early cell cycle proteins required for S phase entry, such as cyclin D1 and RB [178-180].

Since we have found that miR-193a and miR-221 induce DNA synthesis and down-regulation of miR-193a and miR-221 by E2 potentially mediates its anti-mitogenic actions in SMCs, we postulate that they may regulate G<sub>1</sub>/S transition of the cell cycle. Therefore, we further investigated whether miR-193a and miR-221 modulate the expression of genes required for S phase initiation and known to be regulated by E2.

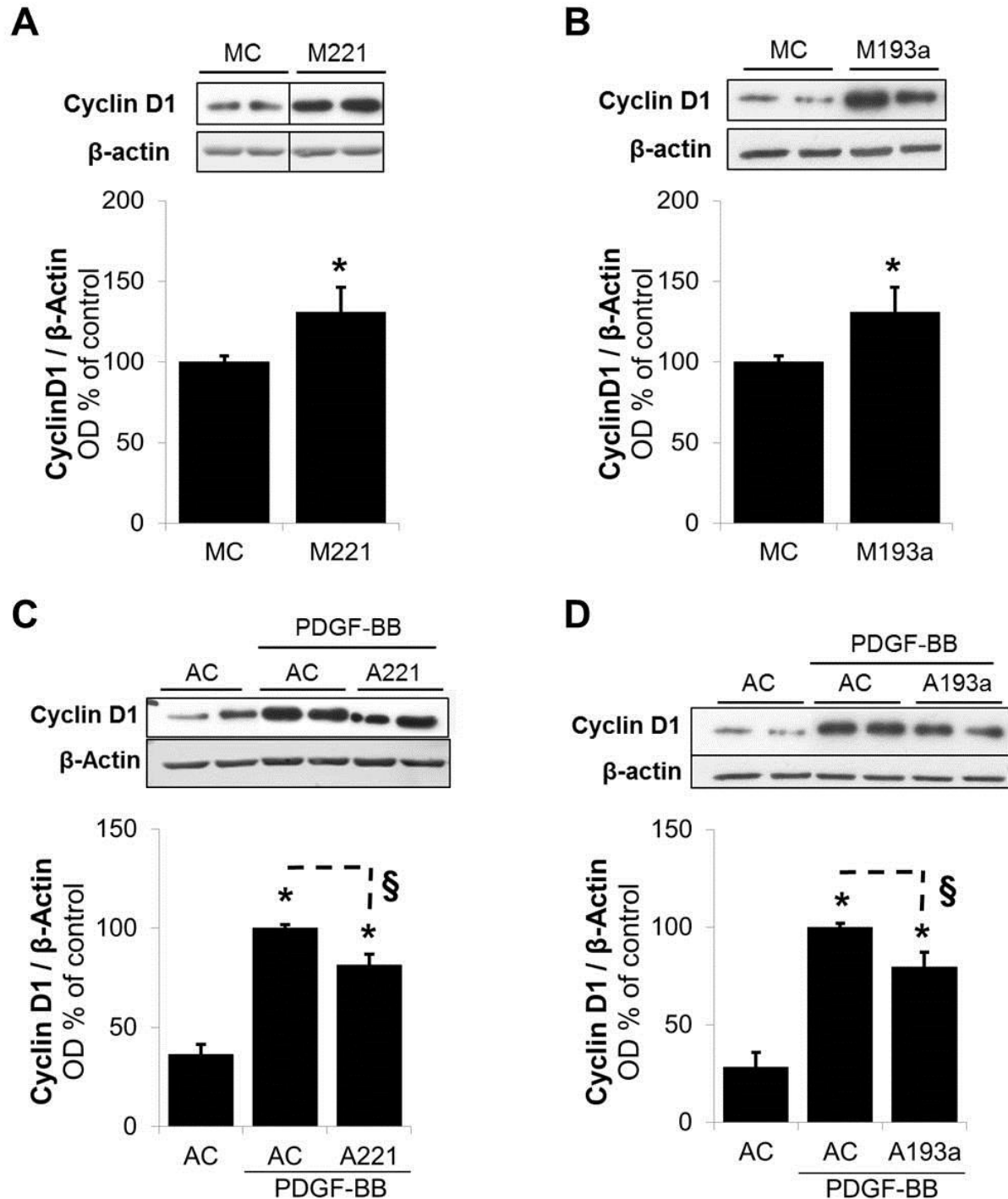
### 5.6.1 miR-221 and miR-193a stimulate G<sub>1</sub>-S phase progression

Cyclin Ds are the first cyclins produced in the cell cycle in response to extracellular signals such as growth factors. In fact, cyclin D1 protein expression was strongly induced in cells treated with PDGF-BB (from 100±7% to 261±21%,  $p<0.05$ ; **Figure 53A**). Treatment with E2 inhibited PDGF-BB-induced cyclin D1 expression in a concentration dependent manner from 176±28% to 135±22% (10 nM) and to 102±21% (100 nM,  $p<0.05$ ; **Figure 53B**).



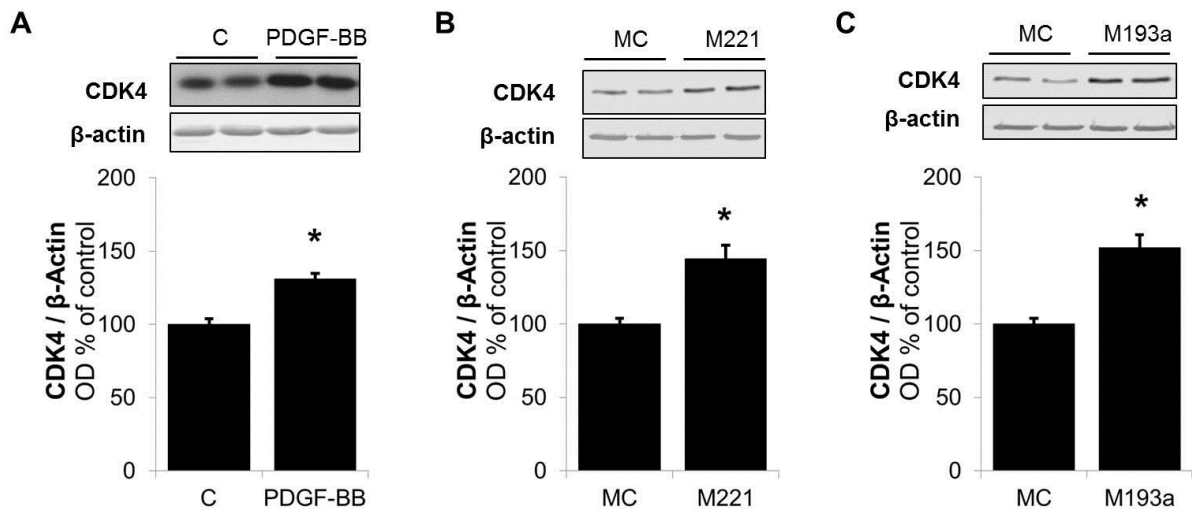
**Figure 53: E2 inhibits PDGF-BB-induced cyclin D1.** Representative Western blot and bar graph of cyclin D1 expression after 48 hr stimulation of HCASMCs with 20 ng/ml PDGF-BB (**Panel A**) and after 1 hr pre-treatment with or without 10 and 100 nM E2 (**Panel B**).  $\beta$ -Actin was used as a loading control.  $n=3$ , \* $p<0.05$  compared to control (C), § $p<0.05$  as indicated.

Similar to PDGF-BB, up-regulation of miR-221 induced cyclin D1 expression in HCASMCs from  $100\pm 9\%$  to  $174\pm 26\%$  ( $p<0.05$ ; **Figure 54A**). Similarly, transfection of the cells with the miR-193a Mimic induced cyclin D1 expression from  $100\pm 9\%$  to  $170\pm 21\%$  ( $p<0.05$ ; **Figure 54B**). Moreover, silencing of both miR-221 and miR-193a using the specific Antimirs significantly abrogated the stimulatory effects of PDGF-BB on cyclin D1, and this effect was analogous to the observed effects of E2. The expression of cyclin D1 was decreased by A221 and A193a by  $19\pm 5\%$  and by  $21\pm 8\%$ , respectively ( $p<0.05$ ; **Figure 53C and 53D**).



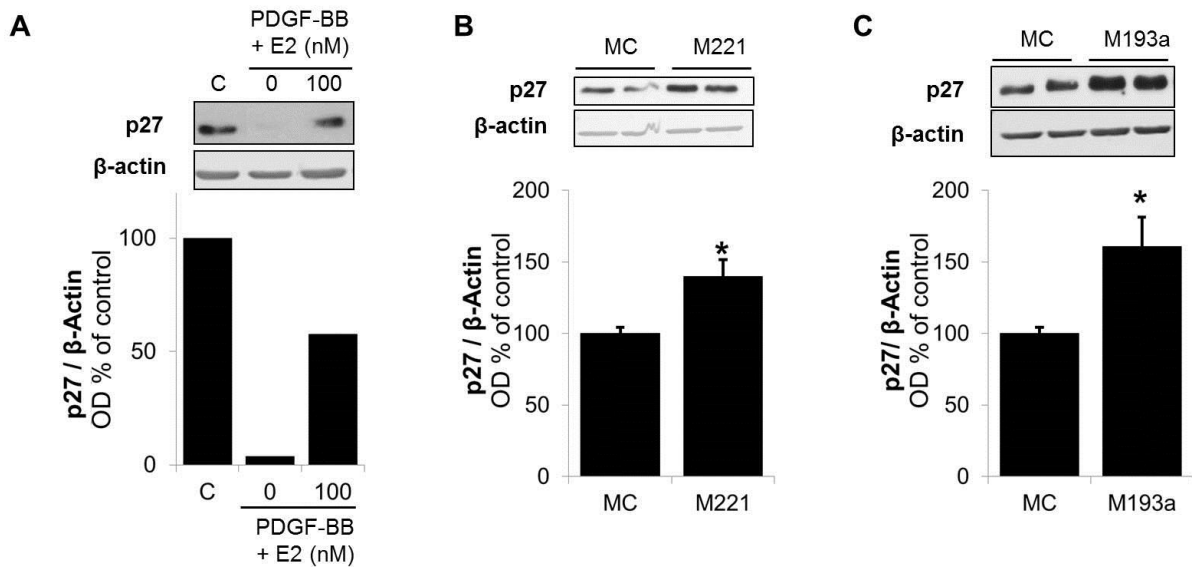
**Figure 54: miR-221 and miR-193a regulate cyclin D1 expression.** Representative Western blot and bar graph of cyclin D1 expression after transfection of the HCASMCs either with Control Mimics (MC), miR-221 Mimic (M221; **Panel A**) or miR-193a Mimic (M193a; **Panel B**) or transfection with the Control Antimir (AC), miR-221 Antimir (A221; **Panel C**) or miR-193a Antimir (A193a; **Panel D**). After transfection, the cells overexpressing miRNAs were kept in starving media for 48 hr prior to lysis, while the cells with down-regulated miRNA were treated 48 hr with 20 ng/ml PDGF-BB.  $\beta$ -Actin was used as a loading control.  $n=3$ , \* $p<0.05$  compared to starved control (C), § $p<0.05$  as indicated.

Even though cyclin D1 levels in proliferating cells are sustained as long as the growth factors are present, cyclin D1 has no effect on G<sub>1</sub>/S transition unless it forms a complex with CDK4 or CDK6. Our findings show that PDGF-BB induced CDK4 protein expression (from 100±4% to 131±4%,  $p<0.05$ ; **Figure 55A**) and that up-regulation of both miR-221 and miR-193a by the specific Mimics had similar significant stimulatory effects (**Figure 55B and 55C**): M221 increased CDK4 from 100±4% to 145±9% and M193a from 100±4% to 152±9%.



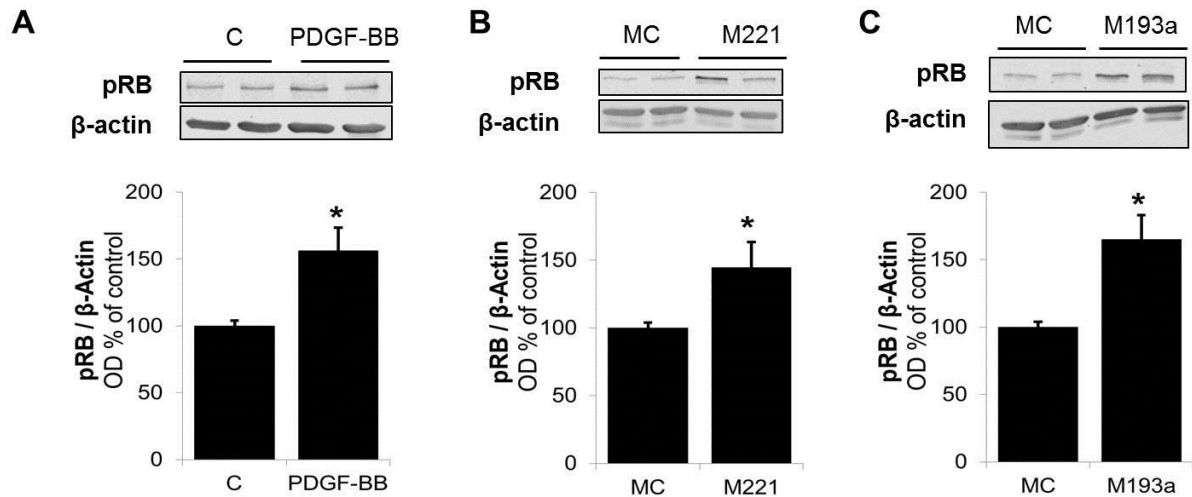
**Figure 55: miR-221 and miR-193a overexpression increases CDK4 expression.** Representative Western blot and bar graph of CDK4 expression after 48 hr treatment with 20 ng/ml PDGF-BB (**Panel A**) or transfection of the HCASMCs with Control Mimics (MC), miR-221 Mimic (M221; **Panel B**) or miR-193a Mimic (M193a; **Panel C**). After transfection the cells overexpressing miRNAs were kept in starving media for 48 hr prior to lysis. β-Actin was used as a loading control.  $n=3$ , \* $p<0.05$  compared to the respective control.

The cyclin-dependent kinase inhibitor 1B (p27) is a negative regulator of the cell cycle progression at G<sub>1</sub>, as it prevents the activation of cyclin D1-CDK4 complexes. PDGF-BB also promotes proliferation by inhibiting p27 (from 100% to 3.6%,  $p<0.05$ ) while E2, consistent with its anti-mitogenic role, reverses its actions (back to 58%,  $p<0.05$  compared to PDGF-BB alone; **Figure 56A**). Surprisingly, both miR-221 and miR-193a Mimics increased the expression of p27 (**Figure 56B and 56C**) from 100±4% to 140±11% and from 100±5% to 161±21%, respectively ( $p<0.05$ ), which is associated with cell quiescence.



**Figure 56: miR-221 and miR-193a overexpression increases p27 expression.** Representative Western blot and bar graph of p27 expression after 48 hr treatment with 20 ng/ml PDGF-BB with or without 100 nM E2 (**Panel A**) or transfection of the HCASMCs with Control Mimics (MC), miR-221 Mimic (M221; **Panel B**) or miR-193a Mimic (M193a; **Panel C**). After transfection the cells overexpressing miRNAs were kept in starving media for 48 hr prior to lysis.  $\beta$ -Actin was used as a loading control.  $n=3$ , \* $p<0.05$  compared to the respective control.

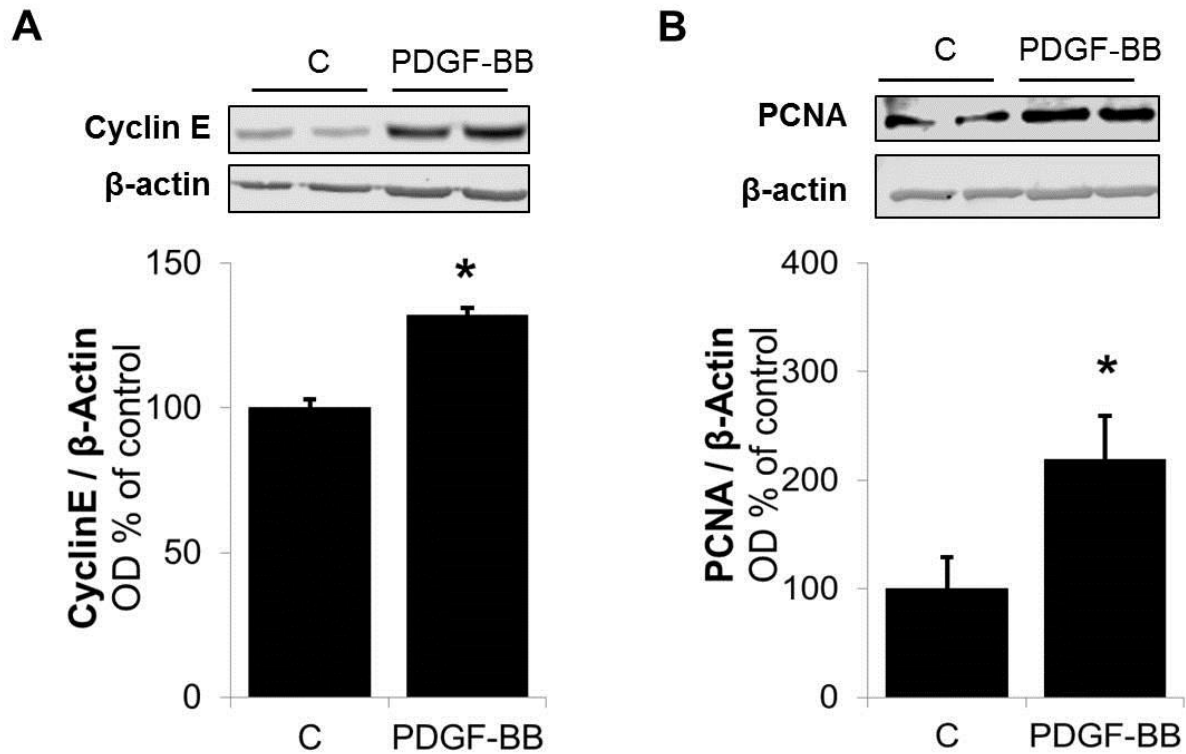
Given the incongruity of the results, increased cyclin D1-CDK4 and p27, we examined the phosphorylation of the retinoblastoma tumor suppressor protein (RB), the principal substrate of CDK4. As a growth factor, PDGF-BB induced hyperphosphorylation of RB from  $100\pm5\%$  to  $130\pm14\%$ , ( $p<0.05$ ; **Figure 57A**). Similarly, up-regulation of miR-221 and miR-193a also increased pRB levels from  $100\pm3\%$  to  $131\pm15\%$  and from  $100\pm4\%$  to  $165\pm18\%$ , respectively ( $p<0.05$ ; **Figure 57B and 57C**), suggesting a growth promoting role in cell cycle progression despite p27 up-regulation.



**Figure 57: miR-221 and miR-193a overexpression increases RB phosphorylation.** Representative Western blot and bar graph of phosphorylated RB (pRB) after 48 hr treatment with 20 ng/ml PDGF-BB (**Panel A**) or transfection of the HCASMCs with the Control Mimic (MC), miR-221 Mimic (M221; **Panel B**) or miR-193a Mimic (M193a; **Panel C**). After transfection the cells overexpressing miRNAs were kept in starving media for 48 hr prior to lysis. n=3, \*p<0.05 compared to the respective control.

Phosphorylation of RB (pRB) by CDK4 allows E2F to dissociate from pRB-E2F complexes, allowing E2F-mediated transcription of cyclins, such as cyclin E and cyclin A, and PCNA, which is essential for DNA replication and S-phase progression. To further test the pro-proliferative role of miR-221 and miR-193a in HCASMCs, we investigated their effect on cyclin E and PCNA expression. We found that, similar to stimulation with PDGF-BB, the transfection of the cells with the Mimics for the two miRNAs did induce both cyclin E and PCNA protein expression. Indeed PDGF-BB stimulated cyclin E and PCNA from 100±3% to 132±3% and 100±29% to 219±40%, respectively (p<0.05; **Figure 58A and 58B**).

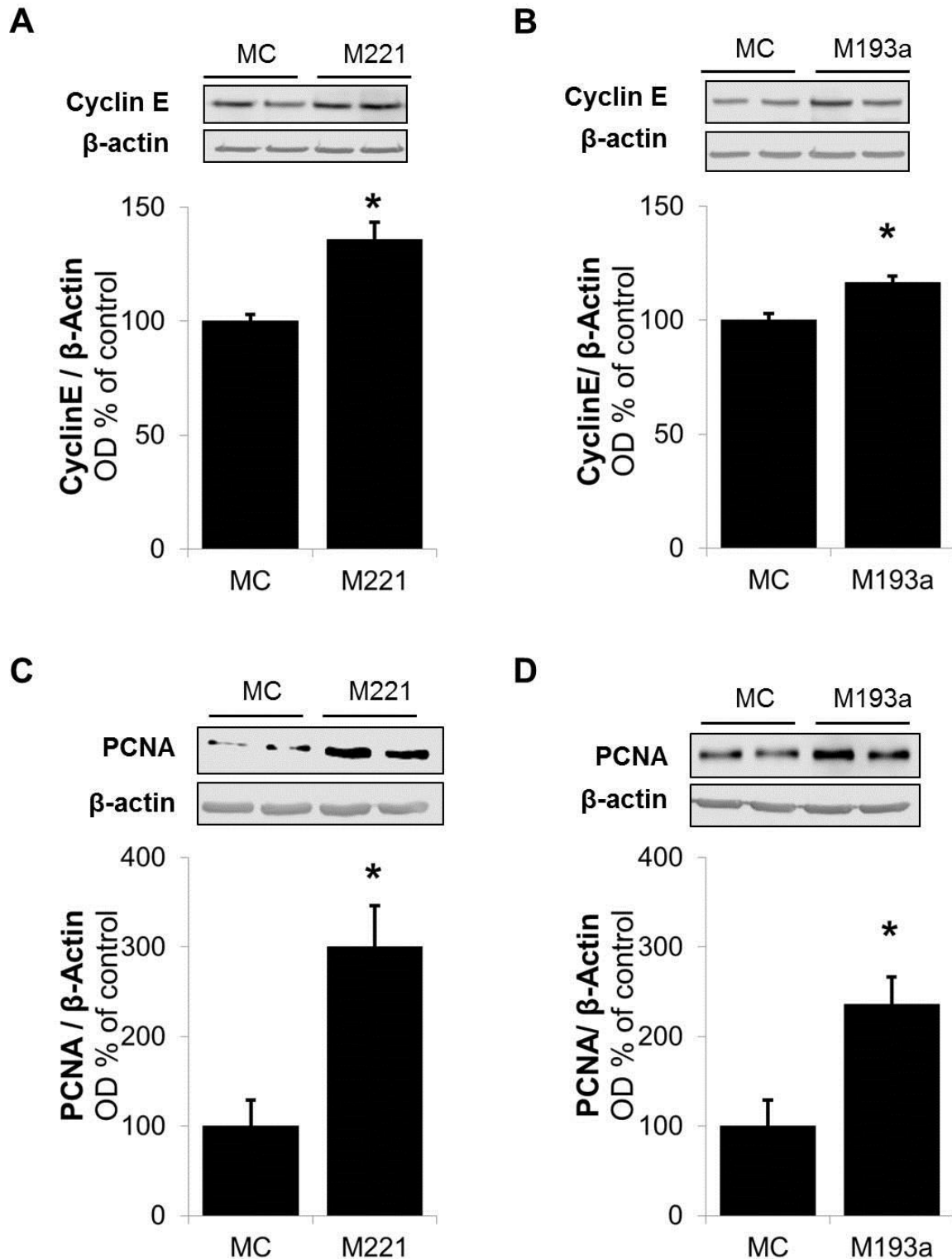




**Figure 58: PDGF-BB promotes S-phase progression through induction of cyclin E and PCNA expression.** Representative Western blot and bar graph of cyclin E (**Panel A**) and PCNA (**Panel B**) expression after 48 hr treatment with 20 ng/ml PDGF-BB. β-Actin was used as a loading control. n=3, \*p<0.05 compared to the respective control.

Up-regulation of miR-221 induced cyclin E expression significantly from 100±3% to 136±7%, whereas miR-193a Mimic showed a smaller induction (from 100±3% to 117±3%, p<0.05; **Figure 59A and 59B**). Similarly, PCNA was significantly up-regulated from 100±29% to 300±46% after transfection with the miR-221 Mimic and from 100±29% to 326±30% with the Mimic for miR-193a (**Figure 59C and 59D**).

These results confirm the pro-proliferative function of the two miRNAs and their role in G<sub>1</sub>-S phase progression. Moreover, they suggest that indeed down-regulation of miR-221 and miR-193a by E2 might mediate its anti-mitogenic actions in SMCs and that this may be mediated via regulation of cell cycle proteins.

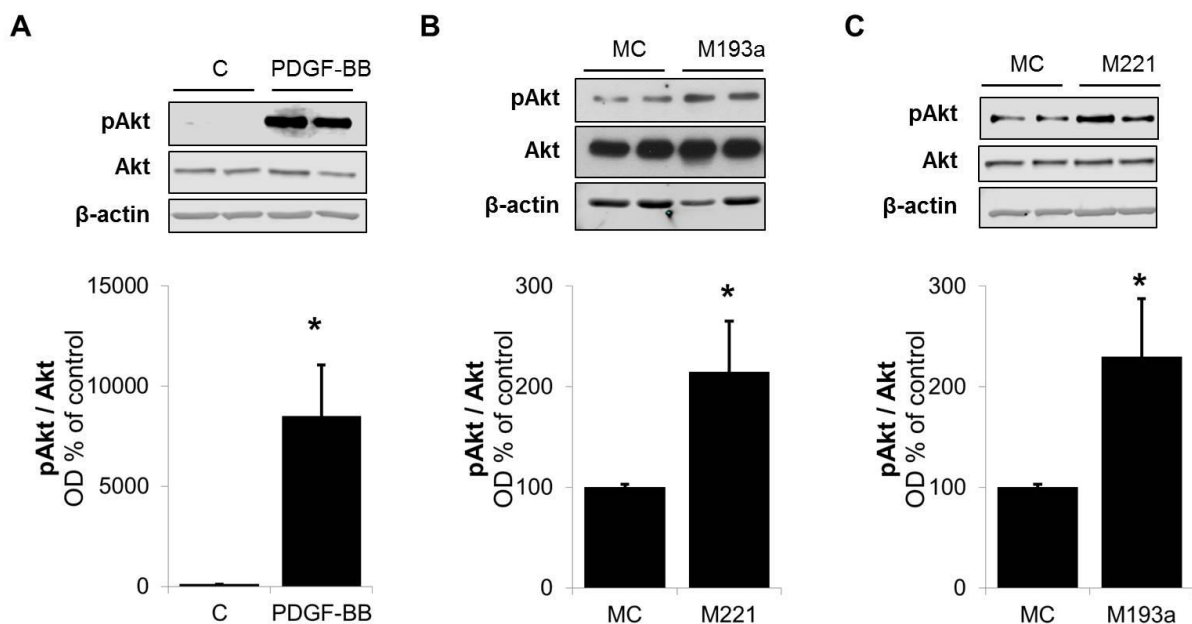


**Figure 59: miR-221 and miR-193a overexpression increases cyclin E and PCNA expression.** Representative Western blot and bar graph of cyclin E and PCNA expression after transfection of the HCASMCs with the Control Mimic (MC), miR-221 Mimic (M221; **Panel A and Panel C**) or miR-193a Mimic (M193a; **Panel B and Panel D**). After transfection, the cells overexpressing miRNAs were kept in starving media for 48 hr prior to lysis. β-Actin was used as a loading control. n=3, \*p<0.05 compared to the respective control.

### 5.6.2 miR-221 and miR-193a activate the PI3K/ Akt signaling pathway

Cell cycle progression is initiated by the binding of growth factors, such as PDGF-BB, to mitogen receptors, which activate the Ras/ Raf/ ERK and PI3K/ Akt signaling pathways. Subsequently, this leads to the activation of transcription factors, such as Myc and AP-1 family members, which in turn alter transcription of genes important in cell cycle, such as *CDK4*, *CDK6* and *cyclin D1* genes.

Since treatment of HCASMCs with PDGF-BB up-regulates miR-193a and miR-221 expression, together with the fact that overexpression of both miRNAs imitates most of the effects of PDGF-BB on the cell cycle, we questioned if they similarly influence the transduction pathways upstream from cell cycle. Indeed, PDGF-BB strongly induced Akt phosphorylation (from  $100\pm3\%$  to  $8478\pm2585\%$ ,  $p<0.05$ ; **Figure 60A**). Moreover, transfection of the cells with the miR-221 and miR-193a Mimic increased Akt phosphorylation from  $100\pm4\%$  to  $215\pm50\%$  and  $100\pm3\%$  to  $229\pm58\%$ , respectively ( $p<0.05$ ; **Figure 60B and 60C**).



**Figure 60: miR-221 and miR-193a promote cell proliferation via the PI3K/ Akt pathway.**

Representative Western blot and bar graph of Akt phosphorylation (pAkt, Ser473) after 48 hr treatment with 20 ng/ml PDGF-BB (**Panel A**) or transfection of the HCASMCs with the Control Mimic (MC), miR-221 Mimic (M221; **Panel B**) or miR-193a Mimic (M193a; **Panel C**). After transfection, the cells overexpressing miRNAs were kept in starving media for 48 hr prior to lysis. Total Akt and  $\beta$ -actin were used as loading controls.  $n=3$ , \* $p<0.05$  compared to the respective control.

### 5.6.3 Discussion

E2 inhibits SMC proliferation, at least in part, by targeting and down-regulating cell cycle progression from G<sub>1</sub> to S phase; hence, here we assessed the effects of miR-193a and miR-221 on the regulation of several early cell cycle proteins.

In the previous chapter (chapter 5.5) we demonstrated that E2 inhibits PDGF-BB-induced DNA synthesis using BrdU incorporation assay. Our findings are consistent with other studies, which showed that PDGF-BB-induced [3H]-thymidine incorporation is significantly decreased after treatment with E2 [174, 175], indicating that E2 might regulate several key molecules required for S phase entry. Indeed, here we provide evidence that E2 inhibits PDGF-BB-induced cyclin D1 expression. Moreover, the suppression of the cyclin-dependent kinase inhibitor p27 by PDGF-BB is reversed by E2, suggesting that E2 causes G<sub>1</sub> arrest of vascular SMCs through inhibition of cyclin D1 and stimulation of p27. In support of our findings, Takahashi et al. has shown that E2 inhibits PDGF-BB-stimulated vascular SMC proliferation by decreasing PDGF-BB-induced cyclin D1 expression and reducing the phosphorylation of RB. However, in contrast to our findings, Takahashi did not observe any changes in the expression of p27 after treatment with E2. Moreover, PDGF-BB-induced CDK4 and CDK2 were also not affected [176]. Although estrogens inhibit proliferation of vascular SMCs, we and other researchers have demonstrated their growth stimulatory effects in ECs [295]. Moreover, estrogens enhance the proliferation of breast and uterine cells [296], indicating that E2 modulates diverse cell functions in a cell and tissue specific way. Nevertheless, modulation of key G<sub>1</sub> phase cell-cycle regulators is an important mechanism by which E2 affects proliferation. In fact, several studies have shown that E2 controls cyclin D1, c-Myc, CDK2, CDK4 and CDK inhibitors in MCF-7 breast cancer cells, thus promoting progression from G<sub>1</sub> to S phase of the cell cycle [297, 298].

We already demonstrated (chapter 5.5) that miR-193a and miR-221 induce DNA synthesis, indicating that these two miRNAs may regulate G<sub>1</sub>/S transition. Moreover, we propose that E2-dependent down-regulation of miR-193a and miR-221 mediates the anti-mitogenic effects of E2. Since E2 arrests vascular SMCs in G<sub>1</sub> phase of the cell cycle, we postulated that miR-193a and miR-221 stimulate cell cycle progression similar to PDGF-BB. Indeed, cyclin D1 expression is stimulated after up-regulation of both miRNAs and down-regulation of both miR-193a and miR-221 decreased PDGF-

BB-induced cyclin D1 expression. Moreover, these miRNAs increased CDK4 expression as well as RB phosphorylation, which is a result of active cyclin D1-CDK4 complexes. Further, we confirmed that E2F is released after miR-193a or miR-221 up-regulation, since both miRNAs induce the expression of E2F targets cyclin E and PCNA. Taken together, our findings reinforce the hypothesis that both miRNAs stimulate cell cycle progression from G<sub>1</sub> to S phase.

There is numerous evidence that miR-221 induces proliferation of vascular SMCs via inhibition of several cell cycle regulators such as p27, p57 and c-Kit [223, 230]; however, our findings provide the first evidence that the mitogenic effects of miR-221 are associated with increased cyclin D1, CDK4, pRB, cyclin E and PCNA in vascular SMCs. Analogously, our findings that miR-193a regulates SMC growth are novel and provide the first evidence of a role for miR-193a in vascular SMCs. Various studies demonstrate that miR-193a inhibits G<sub>1</sub>/S transition and proliferation of melanoma and breast cancer cells by targeting cell cycle proteins, including CDKs, cyclins and p27 [238, 299, 300]. However, we found that miR-193a induces growth of HCASMCs by upregulating cyclin D1, CDK4, pRB, cyclin E and PCNA. These findings indicate that miR-193 modulates the cell cycle progression. Moreover, our assumption that miR-193a positively regulates cell cycle progression is supported by the findings of Wang et al. that up-regulation of miR-193 promotes proliferation of bone mesenchymal stem cells by inducing CDK2 expression by directly targeting its negative regulator, the Inhibitor of Growth Family, Member 5 (ING5) [292].

Although we provide abundant evidence for a pro-proliferative role of miR-193a and miR-221, their up-regulation increased the expression of the cell cycle inhibitor p27. These findings are also in contrast to the results from several studies demonstrating that miR-221 directly targets p27 to inhibit vascular SMC proliferation [222, 223, 230]. Moreover, there is evidence that miR-221 positively regulates the proliferation of several cancer cell types by targeting p27. Gillies et al. have shown that suppression of miR-221 arrested growth of U87 glioblastoma cells via up-regulation of p27 [301]. Galardi et al. observed that knock-down of endogenous miR-221 in PC3 prostate cancer cells blocked proliferation and increased p27 protein, while exogenous expression of miR-221 in LNCaP prostate cancer cells coincided with increased proliferation rates and decreased p27 levels. Moreover, knock-down of p27 after miR-221 suppression rescued the growth arrest in G<sub>1</sub> [302]. The effect of miR-193a on

p27 has solely been studied by Uhlmann et al. His group investigated the impact of different miRNAs, including miR-193a, in EGFR-driven cell cycle pathway and showed that miR-193a up-regulates p27 expression in breast cancer cells [299]. In contrast to vascular SMCs, miR-193a exhibits anti-proliferative actions in breast cancers cells. Moreover, it also inhibits CDK4 and cyclin D1. Therefore we cannot compare our findings on increases in p27 levels with the findings in breast cancer cells, as the growth outcome was different.

Phosphorylation of RB is one of the most crucial steps regulating the progression of the cell cycle as it allows the release of E2F and the subsequent induction of genes required for DNA synthesis. Here we demonstrate that, in spite of high p27 expression, RB is phosphorylated and E2F is probably released, since we observed induction of cyclin E and PCNA expression. These findings support the pro-proliferative role of miR-221 and miR-193a in HCASMCs and suggest that, though p27 levels are high, the molecule may not be functionally active. Since p27 concentration is regulated at different levels, including transcription, translation, protein stability and subcellular localization [303-305], we postulate that one or more of these mechanisms is disrupted by miR-221 and miR-193a overexpression. The regulation of p27 has been shown to differ slightly from one cell type to another [306]. Depending on its phosphorylation, p27 is degraded in the nucleus or exported to the cytoplasm where it is either degraded or retained. In quiescent cells, p27 levels are high and bound to CDK2 to ensure complete inhibition of kinase activity. In proliferating cells, p27 needs to be inactivated in order to allow cyclin E-CDK2 complexes to actively phosphorylate RB and release E2F, thus allowing the transcription of genes important for DNA synthesis in S-phase. Upon mitogenic stimulation, Ras and/or PI3K pathways are activated and trigger c-Myc and Akt to negatively influence p27. c-Myc directly represses p27 expression [307]. Moreover, it induces expression of cyclin Ds and CDK4 so that the cyclin D-CDK4 complexes sequester p27 and CDK2 repression is relieved [307]. This allows CDK2 to phosphorylate p27 at Thr187, making it a target for ubiquitination by the Skp/ Cullin/ F-box complex (SCF<sup>Skp2</sup>) and enhancing p27 degradation in the nucleus [308-310]. Akt directly regulates the expression of p27 through phosphorylation, which results in p27 redistribution and retention in the cytoplasm. Furthermore, Akt indirectly inhibits p27 transcription through inhibition of FoxO transcription factors and maintains cyclin D-CDK complex stability for p27 sequestering [311, 312]. Since c-Myc and Akt are

key molecules in the signaling pathways activating cell proliferation and are required for the transcription of other important cell cycle regulators, it is unlikely that they are inhibited by the pro-proliferative miR-221 and miR-193, thus preventing repression of p27 transcription. Phosphorylation of p27 and its subcellular localization define p27 activity. Therefore it is feasible that miR-221 and miR-193a increase p27 by preventing its suppression, either by enhancing its cytoplasmatic retention or inhibiting its proteosomal degradation. However, this remains speculative and needs to be tested in the future.

In support of our findings, Sherr et al. demonstrate a positive role of p27 in G<sub>1</sub>-phase progression [313]. He discusses the research of different groups demonstrating that the Cip/Kip proteins (p21, p27 and p57) are potent inhibitors of cyclin E- and cyclin A-dependent CDK2, yet they act as positive regulators of cyclin D-dependent kinases [314]. Moreover, Donjerkovic et al. states that p27 and p21 have been shown to associate with CDK/ cyclin complexes both inhibiting or inducing the kinase activity and the stoichiometry is thought to determine which of these final effects are induced [306].

In the current study we observe that miR-221 and miR-193a positively regulate cell cycle progression through up-regulation of cyclin D1, CDK4, pRB, cyclin E and PCNA. However, the direct targets for the two miRNAs remain unclear. Increasing evidence indicates that miRNAs not only repress their targets, but also induce their expression depending on particular cellular conditions, cell type and context [68, 69]. We speculate that miR-193a and miR-221 may target negative regulators of the cell cycle or the mitogenic signal transduction mechanism(s) upstream of cyclin Ds.

The major function of D cyclins is to provide a link between mitogenic stimulus and the cell cycle machinery. Therefore, it is feasible that increased cyclin D1 levels and the downstream cell cycle effectors are merely due to the induction of upstream signaling cascades regulating proliferation processes, such as the Ras/ Raf/ ERK and PI3K/ Akt signaling pathways. Akt promotes G<sub>1</sub>-S phase cell cycle progression by preventing the phosphorylation and degradation of cyclin D1 and  $\beta$ -catenin, which up-regulates the expression of genes important for cell cycle transition, including cyclin D1 [315].

In the current study we investigated the effects of miR-193a and miR-221 on the PI3K/ Akt pathway and demonstrated that both miRNAs activate it in HCASMCs, as

shown by the increase in Akt phosphorylation after miRNA overexpression. However, stimulation of Akt phosphorylation is slight in comparison to the considerable increase after treatment with PDGF-BB, suggesting that this is not the main mechanism by which miR-193a and miR-221 induce vascular SMC proliferation. Alternatively, the potent effects of PDGF-BB may be due to a combination of factors beside miR-221 and miR-193a.

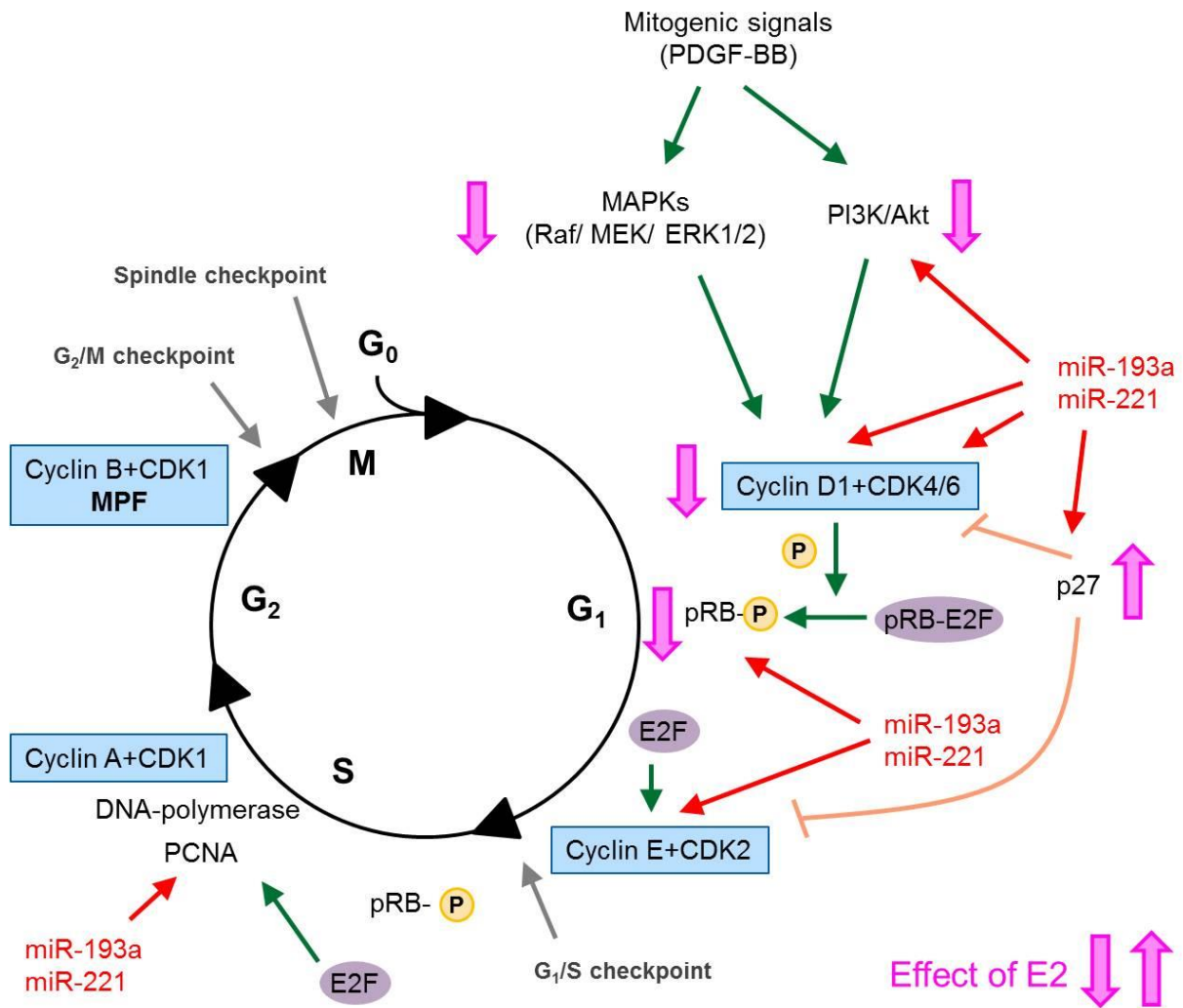
Besides regulation of PI3K/ Akt, the anti-mitogenic actions of E2 include inhibition of Raf/ Mek/ Erk MAPK signaling, which plays a prominent role in proliferation. Thus, based on the fact that E2 inhibits SMC growth by abrogating MAPK phosphorylation [178], we speculate that miR-193a and miR-221 may contribute to vascular SMC growth also through induction of this MAPK pathway.

We already discussed the role of miR-221 and miR-193a on the PI3K/ Akt pathway in a previous chapter (chapter 5.3). In contrast to HUVECs, where both miRNAs inhibit PI3K/ Akt signaling, in HCASMCs they activate it. In support of these findings, miR-221 is shown to induce PI3K/ Akt signaling by suppressing the negative regulator PTEN in several cancer cell lines [273-275]. Further, miR-193a indirectly inhibits PTEN, thus activating Akt and contributing to leukemogenesis [257] and suppresses the metastasis of human non-small-cell by indirect inhibition of PI3K/ Akt cascade [271]. Therefore it is possible that miR-193a and miR-221 increase PI3K/ Akt signaling in HCASMCs through targeting its negative regulator PTEN. Taken together, these findings strongly suggest that miR-193a and miR-221 differentially regulate the PI3K/ Akt signaling pathway depending on the cell type and context.

In summary, in this chapter we first demonstrate that E2 inhibits PDGF-BB-induced vascular SMC proliferation via inhibition of cell cycle progression from G<sub>1</sub> to S phase. Specifically, E2 decreased PDGF-BB-induced cyclin D1 expression and up-regulated the cell cycle inhibitor p27. Moreover, we provide insight into the molecular mechanisms by which miR-193a and miR-221 influence vascular SMC proliferation (**Figure 61**). Overexpression of both miRNAs induces Akt phosphorylation, indicating that they activate the PI3K/ Akt pathway. Similar to PDGF-BB, up-regulation of both miRNAs induced cyclin D1, CDK4, cyclin E and PCNA expression and increased RB phosphorylation. However, in contrast to our expectations and to the results from other researchers, both miRNAs also induced p27. Nevertheless, we found that both miRNAs induced PCNA expression and promoted DNA synthesis (results from the



previous chapter), thus it is likely that p27 up-regulation is not associated with increased p27 activity. In conclusion, the mechanisms by which miR-221 and miR-193a regulate cell cycle progression still need to be elucidated to provide a better understanding of how down-regulation of these miRNAs by E2 mediates the anti-mitogenic actions of E2 in vascular SMCs.



**Figure 61: Summary of our findings in a simplified schematic representation of the cell cycle in vascular SMCs and its regulation by E2.** G<sub>0</sub>, quiescence; G<sub>1</sub>, Gap 1; S, Synthesis; G<sub>2</sub>, Gap 2; M, Mitosis; PDGF-BB, Platelet-Derived Growth Factor BB; MAPK, Mitogen Activated Protein Kinase; PI3K, Phosphoinositide 3-Kinase; CDK, Cyclin-Dependent Kinase; RB, Retinoblastoma protein; PCNA, Proliferating Cell Nuclear Antigen; MPF, Mitosis Promoting Factor.

## 5.7 ER $\alpha$ mediates miR-221 and miR-193a down-regulation

### Objective

The role of ERs in mediating the beneficial effects of E2 in vascular cells is well established. Therefore, under this specific aim, we examined whether down-regulation of miR-193a and miR-221 by E2 is mediated via ER $\alpha$ , ER $\beta$  or GPER.

### Introduction

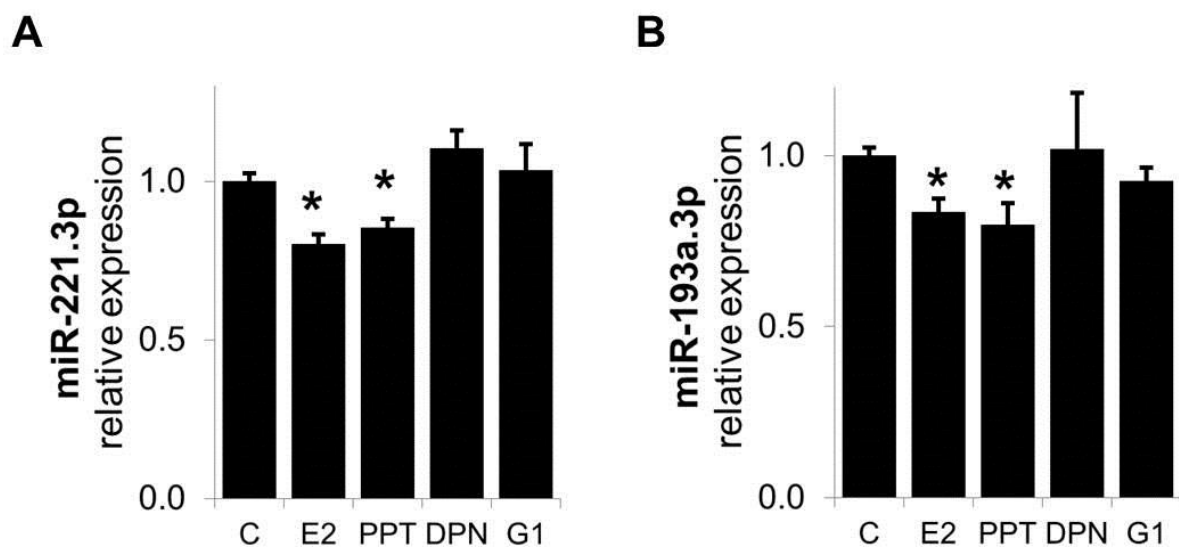
The biologic actions of E2 are mediated via genomic pathways, which involve changes in gene expression either through direct activation of the ERs as transcription factors, via association with other transcription factors or without the involvement of estrogens [44]. Additionally, E2 can also induce its biological effects via rapid non-genomic mechanisms [35], most likely initiated at the membrane level, which include the activation of intracellular signaling cascades. The genomic pathways are mediated via the classic ERs, ER $\alpha$  and ER $\beta$ , whereas the rapid non-genomic mechanisms presumably involve ER $\alpha$  [40] and GPER [29, 32] at the membrane level.

E2-dependent modulation of miR-193a and miR-221 is potentially mediated via genomic or non-genomic mechanisms and might involve one or more ERs. Indeed, all three ERs have been shown to directly or indirectly regulate miRNAs in tumors and normal cells. For example, ER $\alpha$  mediates the down-regulation of miR-21, miR-26a, miR-143, miR-145, miR-195 and miR-221, while it stimulates the expression of miR-19, miR-101, miR-203 and miR-425. ER $\beta$  is associated with inhibition of miR-17, miR-24, miR-183 and miR-301a, and up-regulation of miR-23b, miR-27b and miR-205. GPER was shown to mediate repression of miR-338 [316].

Therefore, we investigated the role of ERs in mediating the down-regulation of miR-193a and miR-221 in HUVECs and HCASMCs. To achieve this, we used pharmacologically specific and unspecific ER agonists and antagonists. The specific agonists include PPT (ER $\alpha$ ), DPN (ER $\beta$ ) and G1 (GPER). The antagonists include ICI 182,780 (ICI, unspecific ER inhibitor), MPP (specific for ER $\alpha$ ), PHTPP (specific for ER $\beta$ ) and G15 (GPER inhibitor).

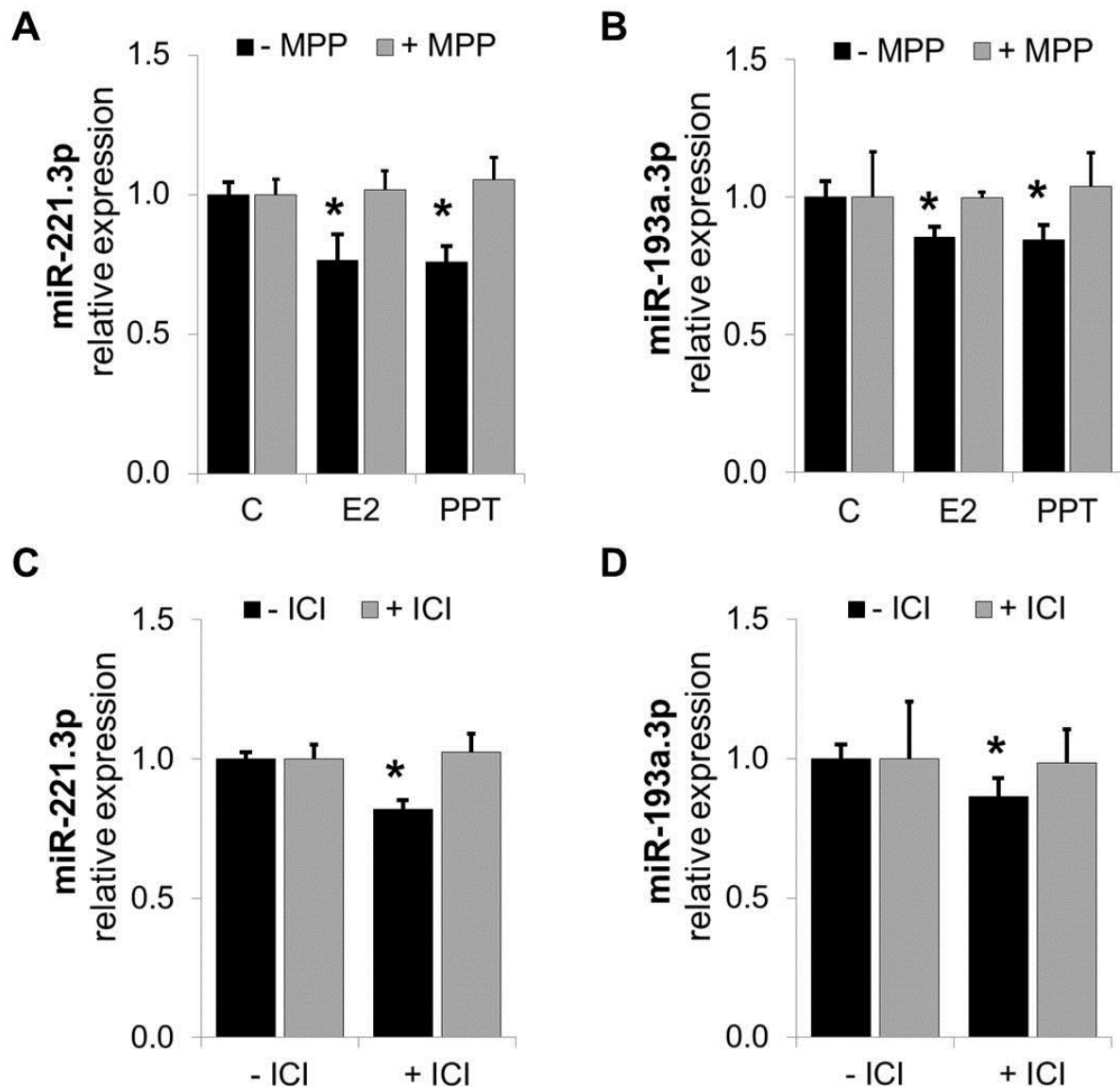
### 5.7.1 ER $\alpha$ mediates miRNA down-regulation in HUVECs

Treatment of HUVECs with E2 and the Estrogen Receptor (ER) specific agonists revealed a role for ER $\alpha$  in mediating the suppressive effects of E2 on miR-221 and miR-193a expression. In fact, E2 and the ER $\alpha$  agonist PPT decreased miR-221 expression by  $20\pm3\%$  and  $15\pm3\%$  respectively ( $p<0.05$ ) and miR-193a expression by  $17\pm4\%$  and  $21\pm6\%$  respectively ( $p<0.05$ ). Besides, ER $\beta$  and GPER agonists DPN and G1 did not significantly alter the expression of the two miRNAs, which changed from  $100\pm2\%$  to  $102\pm16\%$  and  $92\pm4\%$ , respectively, for miR-221 and from  $100\pm3\%$  to  $110\pm6\%$  and  $103\pm8\%$ , respectively, for miR-193a (**Figure 62A and 62B**).



**Figure 62: ER $\alpha$  agonist PPT down-regulates miR-193a and miR-221 expression in HUVECs.** Cells were grown to 60% confluency in complete media prior to 24 hr treatment with E2 (10 nM) and the ER agonist: PPT (100 nM, ER $\alpha$  agonist), DPN (100 nM, ER $\beta$  agonist), G1 (100 nM, GPER agonist). Total RNA was extracted and relative miRNA expression levels were determined by RT-qPCR using TaqMan miRNA assays for miR-221.3p (**Panel A**) and miR-193a.3p (**Panel B**). The results were normalized to U48 and U49.  $n=3$ , \* $p<0.05$  compared to control.

The importance of ER $\alpha$  is further confirmed by addition of the ER $\alpha$  antagonist MPP, which reversed the inhibitory effect of E2 and PPT on both miR-221 and miR-193a (**Figure 63A and 63B**). As a positive control, the down-regulation of the two miRNAs by E2 is reversed by the unspecific ER antagonist ICI 182780 (**Figure 63C and 63D**). These observations indicate that ER $\alpha$  is necessary for E2-mediated down-regulation of miR-221 and miR-193a.

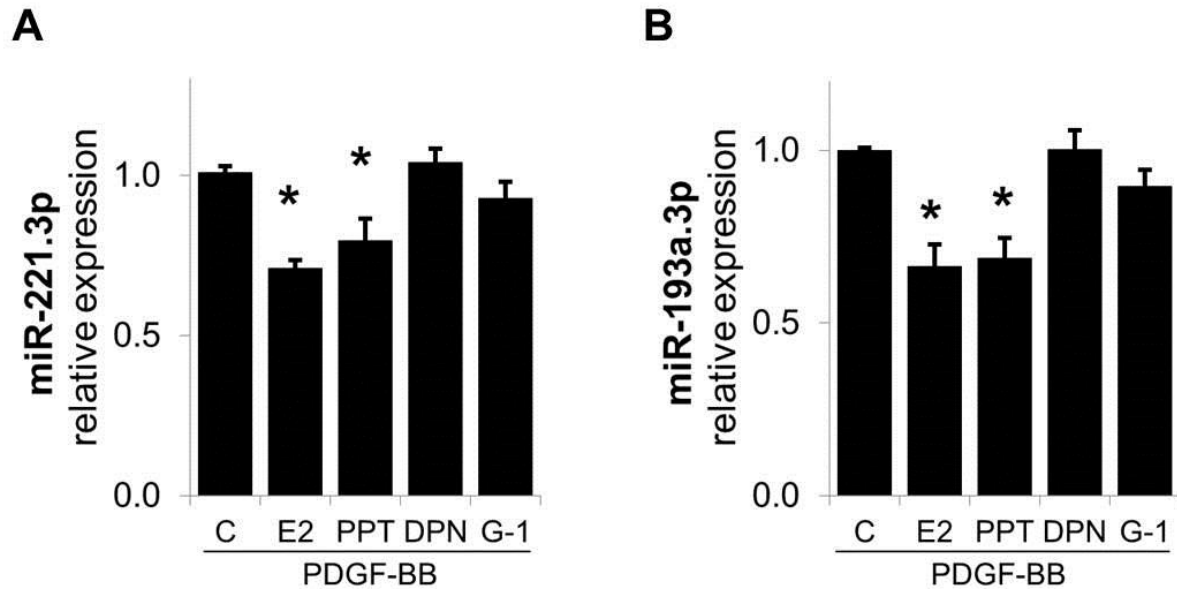


**Figure 63: ER $\alpha$  mediates down-regulation of miR-193a and miR-221 expression in HUVECs.** Cells were grown to 60% confluency in complete media prior to 24 hr treatment with E2 (10 nM), PPT (100 nM, ER $\alpha$  agonist) and the ER antagonist MPP (500 nM, ER $\alpha$  antagonist) and ICI (1  $\mu$ M, ER $\alpha$  and ER $\beta$  antagonist). Total RNA was extracted and relative miRNA expression levels were determined by RT-qPCR using TaqMan miRNA assays for miR-221.3p (**Panel A and C**) and miR-193a.3p (**Panel B and D**). The results were normalized to U48 and U49. n=3, \*p<0.05 compared to control, §p<0.05 as indicated.

### 5.7.2 ER $\alpha$ mediates miRNA down-regulation in HCASMCs

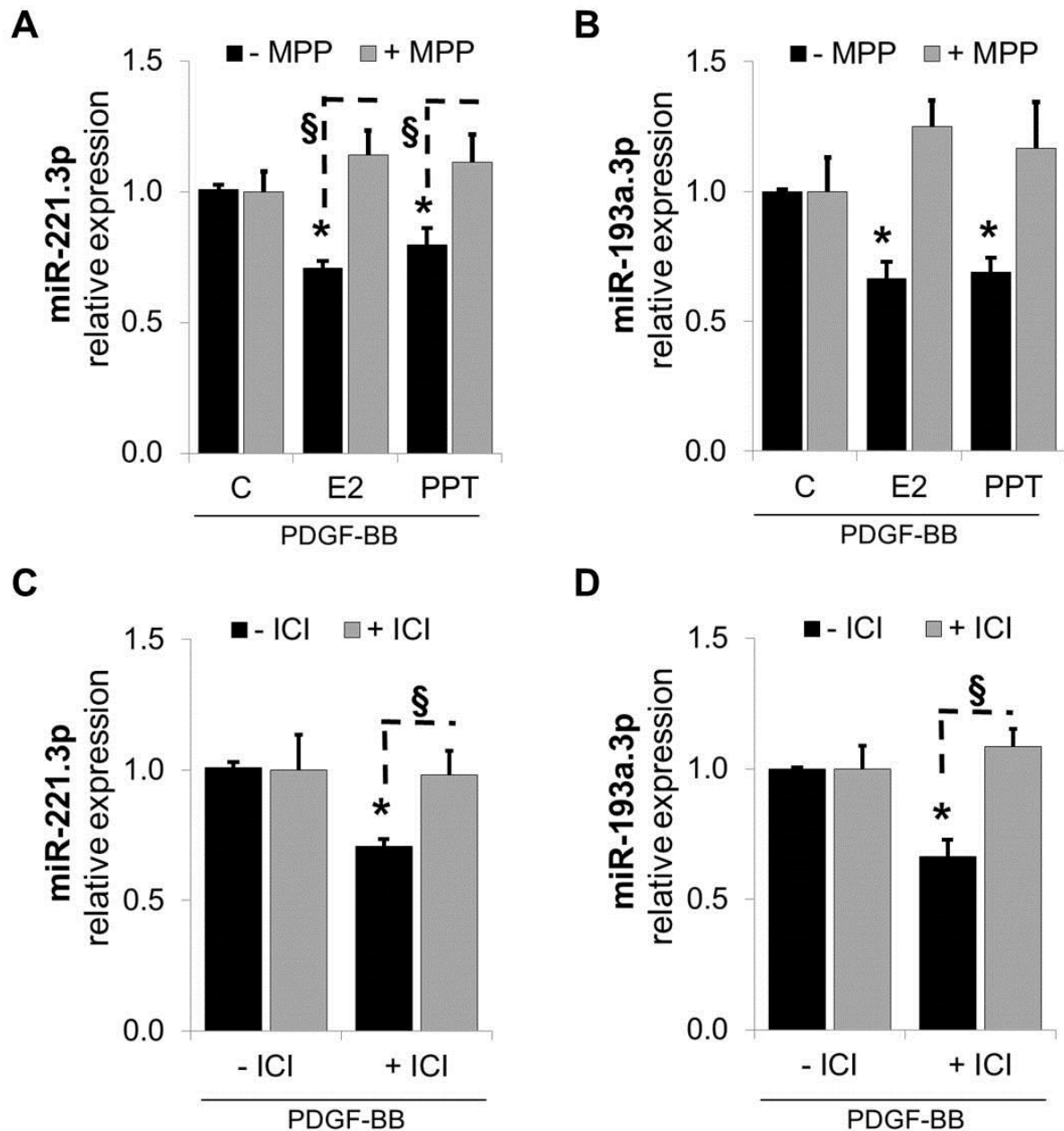
The inhibitory effects of E2 on PDGF-BB-induced miR-221 production in HCASMCs were mimicked by the ER $\alpha$  agonist PPT, but not by ER $\beta$  and GPER agonists (**Figure 64A**). E2 and PPT decreased miR-221 expression by  $29\pm3\%$  and by  $20\pm7\%$  respectively (p<0.05). Similar results were seen for PDGF-BB-induced miR-193a.

Both E2 and the ER $\alpha$  agonist PPT down-regulated miR-193a expression by  $34\pm6\%$  and  $31\pm6\%$ , respectively ( $p<0.05$ ), while ER $\beta$  and GPER agonists had no statistically significant effect (**Figure 64B**).



**Figure 64: ER $\alpha$  agonist PPT down-regulates miR-193a and miR-221 expression in HCASMCs.** Cells were grown to 60% confluency in complete media prior to 24 hr treatment with PDGF-BB (20 ng/ml), E2 (10 nM) or the ER agonist: PPT (100 nM, ER $\alpha$  agonist), DPN (100 nM, ER $\beta$  agonist), G1 (100 nM, GPER agonist). Total RNA was extracted and relative miRNA expression levels were determined by RT-qPCR using TaqMan miRNA assays for miR-221.3p (**Panel A**) and miR-193a.3p (**Panel B**). The results were normalized to U48 and U49.  $n=3$ , \* $p<0.05$  compared to control.

Furthermore, 500 nM of the ER $\alpha$  antagonist MPP significantly blocked the effects of both E2 and PPT, thus inhibiting down-regulation of both miR-221 and miR-193a (**Figure 65A and 65B**). The unspecific ER antagonist ICI 182780 (1  $\mu$ M) was used as a positive control and also blocked miR-221 and miR-193a down-regulation by E2 (**Figure 65C and 65D**). These results suggest a role for ER $\alpha$  in mediating the modulation of miR-221 and miR-193a.



**Figure 65: ER $\alpha$  mediates down-regulation of miR-193a and miR-221 expression in HCASMCs.** Cells were grown to 60% confluency in complete media prior to 24 hr treatment with PDGF-BB (20 ng/ml), E2 (10 nM), PPT (100 nM, ER $\alpha$  agonist) and the ER antagonist MPP (500 nM, ER $\alpha$  antagonist) and ICI (1  $\mu$ M, ER $\alpha$  and ER $\beta$  antagonist). Total RNA was extracted and relative miRNA expression levels were determined by RT-qPCR using TaqMan miRNA assays for miR-221.3p (**Panel A and C**) and miR-193a.3p (**Panel B and D**). The results were normalized to U48 and U49. n=3, \*p<0.05 compared to control.

### 5.7.3 Discussion

The role of ERs in mediating the beneficial effects of E2 in vascular cells is well established. Therefore, we investigated whether they are also involved in the down-regulation of miR-193a and miR-221 by E2 in HUVECs and HCASMCs.

Here we show that down-regulation of the two miRNAs is mimicked by the ER $\alpha$  agonist PPT and not by ER $\beta$  or GPER agonists. Moreover, the broad spectrum ER antagonist ICI and the ER $\alpha$  specific antagonist MPP blocked E2- and PPT-dependent miRNA inhibition, suggesting that ER $\alpha$  is required for E2-mediated miRNA down-regulation.

Numerous studies report that ER $\alpha$  mediates the cardiovascular protective effects of estrogen. In ER $\beta$  knock-out mice, estrogen was still protective against vascular injury [317], whereas in ER $\alpha$  knock-out mice estrogen treatment showed no protective effect on vascular SMC proliferation after vascular injury [318], supporting that ER $\alpha$  is necessary for the anti-mitogenic actions of estrogen in vascular SMC. Moreover, Takahashi et al. reported that the inhibitory effects of E2 on RB phosphorylation are lost in the presence of the unspecific antagonist ICI. Moreover, E2 could not inhibit PDGF-BB-induced proliferation in A10 cells (a primary rat embryonic thoracic aorta cell line) lacking ER $\alpha$  expression, but significantly inhibited the PDGF-BB-induced cyclin D1 in A10 cells transfected with ER $\alpha$ , suggesting that ER $\alpha$  is required for E2-induced G<sub>1</sub> arrest of vascular SMCs [180]. Finally, ER $\alpha$  mediates the rapid E2 effects leading to increased Akt phosphorylation in vascular SMCs and ECs [191] and is required for the activation of eNOS and E2-dependent vascular relaxation in ECs [41, 319].

Although there is ample evidence for a role of ER $\alpha$  in mediating the vaso-protective actions of E2, which ER is responsible for the beneficial effect of E2 in the vascular system is still intensely debated. The results from Watanabe et al. indicate that ER $\beta$  is more potent than ER $\alpha$  in the inhibitory effect on vascular SMC proliferation [320]. Hodge et al. reported that ER $\beta$  is the predominantly expressed form of ER in vascular SMCs, suggesting that the protective effects of estrogens in the cardiovascular system may be due to the genomic effects of ER $\beta$  in vascular tissue [321]. In addition, there is evidence that estrogen inhibits vascular SMC proliferation in response to vascular injury in knock-out mice lacking ER $\alpha$  [322]. However, ER $\alpha$  may be involved in the protective effects because this knock-out mouse expressed a

variant ER $\alpha$  [323]. Finally, studies with ER $\alpha$ / ER $\beta$  double knock-out mice demonstrated decreased proliferation of vascular SMCs upon estrogen treatment, suggesting a role also for GPER in mediating the beneficial actions of estrogen on the vascular system [324].

In support of our findings that ER $\alpha$  is required for down-regulation of miR-193a and miR-221, E2 and the ER $\alpha$ -selective agonist PPT decreased miR-206 expression in MCF-7 cells [325]. Moreover, Di Leva et al. reported that E2-ER $\alpha$  mediate the repression of miR-221/222 in MCF-7 cells [291] and Zhao et al. demonstrated that ER $\alpha$  mediates miR-203 up-regulation by E2 in vascular SMCs and that miR-203 participates in the anti-mitogenic actions of E2 in these cells [94].

Interestingly, miRNAs have also been reported to influence E2-regulated gene expression by directly reducing ER $\alpha$  mRNA stability or translation [84, 86, 316, 326]. The role of miR-221/222 in breast cancer resistance has been extensively studied. Indeed, these miRNA are both down-regulated by E2-ER $\alpha$  and in turn target ER $\alpha$ , pointing to a regulatory loop between ER $\alpha$  and miRNA expression [291, 327-329].

In summary, here we provide evidence that ER $\alpha$  is involved in miR-193a and miR-221 regulation; however, we did not investigate whether these miRNAs influence ER $\alpha$  expression in vascular cells. Furthermore, the molecular mechanisms by which E2-ER $\alpha$  inhibit miR-193a and miR-221 expression remain unknown.



## 5.8 E2 modulates miR-221 and miR-193a processing

### Objective

We demonstrated that ER $\alpha$  is required for the down-regulation of miR-193a and miR-221 in HUVECs and HCASMCs. Since E2-ER $\alpha$  regulates gene expression including miRNA expression, we hypothesize that E2 inhibits miR-193a and miR-221 at the transcriptional level. Hence, we examined the expression of pri-miRNAs following E2 treatment.

### Introduction

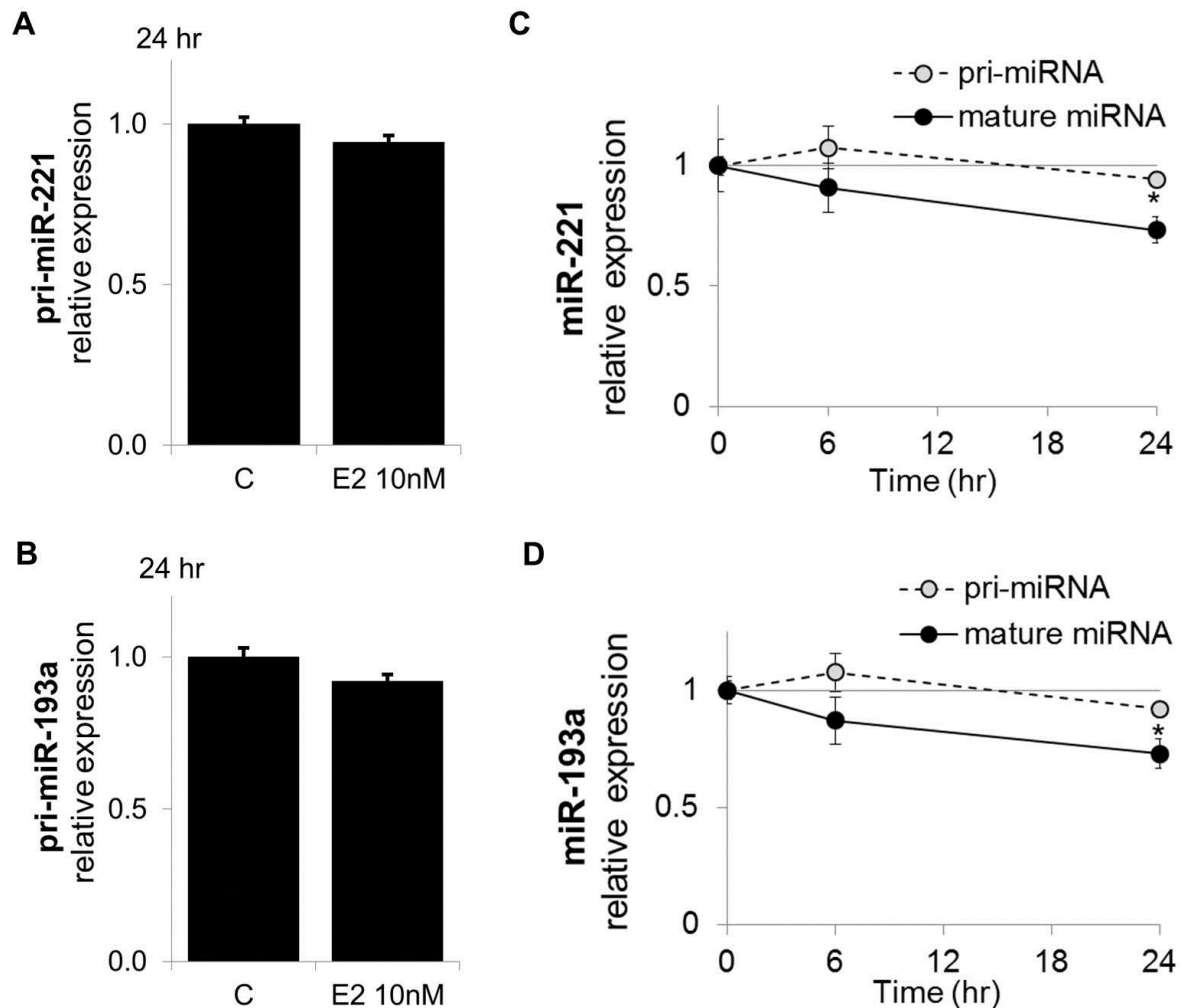
Our results from section 5.7 show that down-regulation of miR-193a and miR-221 by E2 requires ER $\alpha$  in both HUVECs and HCASMCs. ER $\alpha$  is a ligand-activated transcription factor, which mediates the effects of E2 either via alteration of gene expression or activation of rapid intracellular transduction pathways. Biogenesis of miRNAs is regulated at multiple levels, including modulation of miRNA gene transcription and procession to mature miRNAs by Drosha and Dicer in the nucleus and cytoplasm, respectively. Moreover, AGO2 loading and RNA decay also modulate miRNA activity and expression [59]. Many miRNAs are regulated directly by transcription factors, as their promoter region is similar to that of regular protein coding genes. E2-ER $\alpha$  has also been shown to alter miRNA expression. Indeed, there is evidence for ER $\alpha$  mediated regulation of miRNA at the transcriptional level. Zhao et al. demonstrated that E2-ER $\alpha$  induces miR-203 transcription in vascular SMC [94]. Di Leva et al provide evidence that E2-ER $\alpha$  directly represses miR-221 transcription in MCF-7 cells [291].

Based on the above evidence, we hypothesize that E2 inhibits miR-193a and miR-221 at the transcriptional level. To test this hypothesis, using RT-qPCR, we examined the expression of the pri-miRNAs in both HUVECs and HCASMCs treated with E2.

### 5.8.1 E2 does not influence pri-miR-221 and pri-miR-193a in HUVECs

Treatment of HUVECs with 10 nM E2 for 24 hr only slightly diminished the expression of both pri-miR-221 (from 100 $\pm$ 2% to 94 $\pm$ 2%, n.s.) and pri-miR-193a (from 100 $\pm$ 3% to 92 $\pm$ 2%, n.s.; **Figure 66A and 66B**). To exclude the possibility of time-dependent down-regulation of the pri-miRNA by E2, we examined their expression also after 6 hr of treatment. As shown in **Figure 66C**, we found that pri-

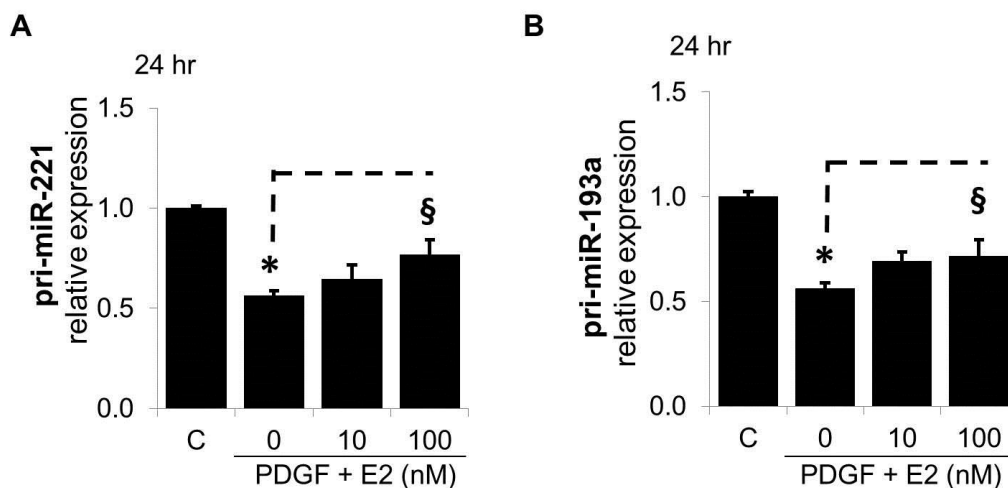
miR-221 is not affected by E2 (relative expression of  $107 \pm 9\%$ , n.s.). Likewise, pri-miR-193a expression did not change after 6 hr E2 treatment (from  $100 \pm 6\%$  to  $107 \pm 8\%$ ) and is only slightly reduced after 24 hr treatment by  $8 \pm 2\%$  (n.s.; **Figure 66D**).



**Figure 66: E2 does not inhibit pri-miRNA expression in HUVECs.** Cells were grown to 60% confluency in complete media prior to treatment with or without E2 (10 nM). **Panel A and B:** Pri-miR-221 and pri-miR-193a expression after 24 hr treatment with E2. **Panel C and Panel D:** Comparison of pri-miRNA and mature miRNA expression after 6 and 24 hr treatment. Total RNA was extracted and relative pri-miRNA expression levels were determined by RT-qPCR using TaqMan assays for pri-miR-221 and pri-miR-193a. The results were normalized to GAPDH and hPRT1 mRNA. n=3, \*p<0.05 compared to control.

### 5.8.2 E2 abrogates the effects of PDGF-BB on pri-miRNA expression in HCASMCs

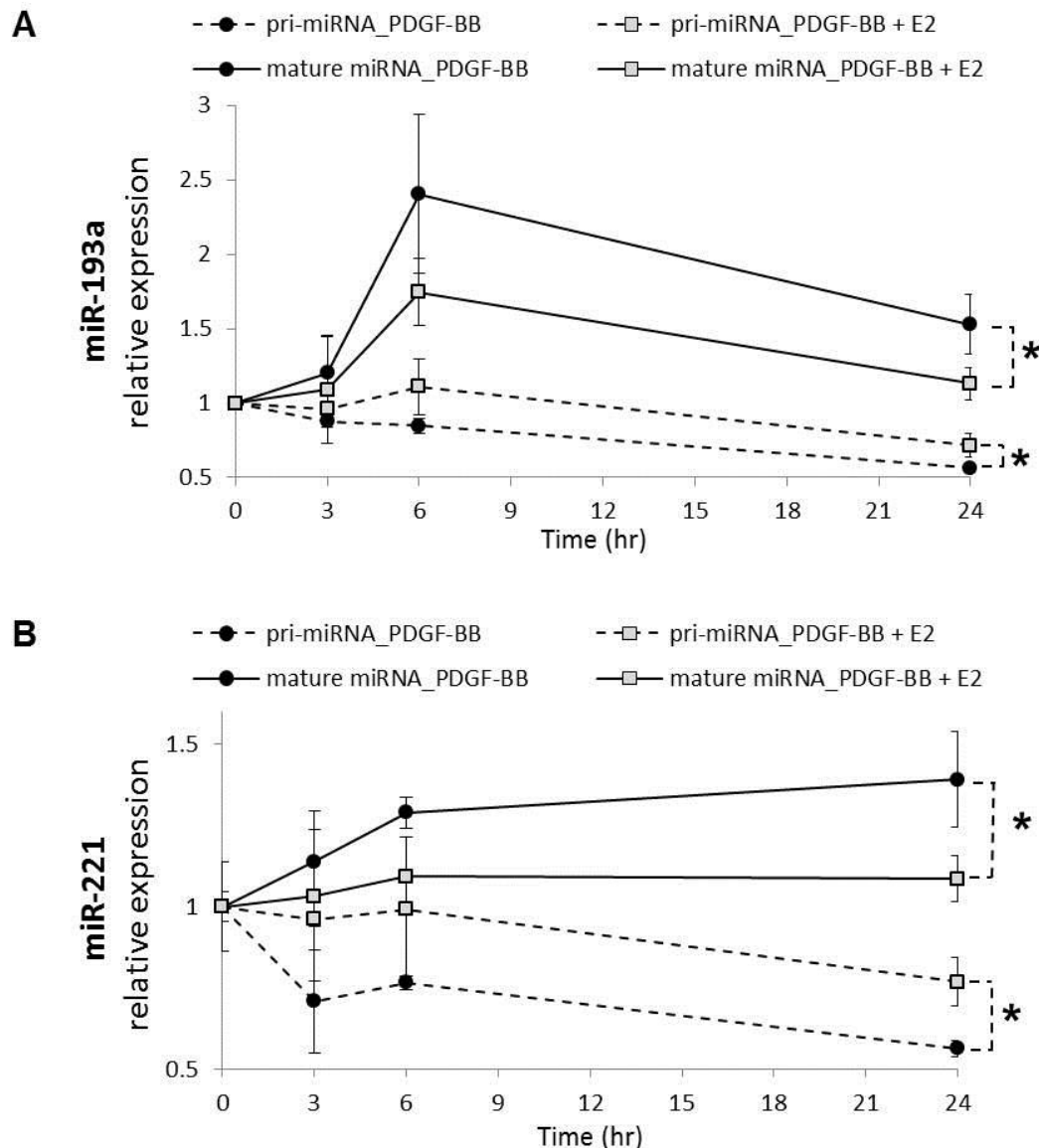
In HCASMCs the expression of the pri-miRNAs was strongly affected by 24 hr treatment with PDGF-BB and the effect of PDGF-BB was significantly reversed by treatment of the cells with 100 nM E2 (**Figure 67A and 67B**). Indeed, PDGF-BB reduced pri-miR-221 (by  $43\pm3\%$ ,  $p<0.05$ ), while E2 increased it in a concentration dependent manner from  $57\pm3\%$  to  $64\pm7\%$  and  $77\pm7\%$ , respectively ( $p<0.05$ ). Similarly, pri-miR-193a was inhibited by stimulation of the cells with PDGF-BB (by  $44\pm2\%$ ,  $p<0.05$ ). Moreover, the inhibitory-effect of PDGF-BB was abrogated in a concentration-dependent manner by E2 (from  $56\pm2\%$  to  $69\pm4\%$  and  $71\pm8\%$ , respectively ( $p<0.05$ ).



**Figure 67: E2 reversed the PDGF-BB-dependent decrease in pri-miR-221 and pri-miR-193a in HCASMCs.** Cells were grown to 60% confluency in complete media prior to treatment with PDGF-BB and E2 (0, 10 and 100 nM). **Panel A and B:** Pri-miR-221 and pri-miR-193a expression after 24 hr treatment with E2. Total RNA was extracted and relative pri-miRNA expression levels were determined by RT-qPCR using TaqMan assays for pri-miR-221 and pri-miR-193a. The results were normalized to GAPDH and hPRT1 mRNAs.  $n=3$ ,  $*p<0.05$  compared to control,  $§p<0.05$  as indicated.

Since the observed effect might be due to the conversion of pri-miRNAs into mature miRNAs, we additionally looked at the pri-miRNA expression after 3 and 6 hr treatment with E2 and compared it with the expression of the mature miRNAs. Interestingly, the levels of pri-miR-221 decreased in response to PDGF-BB at each tested time point, whereas mature miR-221 was induced. Moreover, E2 lessened the effects of PDGF-BB in regard to both the pri- and the mature miRNA (**Figure 68A**). In

regard to miR-193a we observed similar results as for miR-221: the pri-miR-193 is decreased by PDGF-BB at each tested time, while the mature miR-193a is strongly induced. Again, E2 treatment reversed the effect of PDGF-BB on both pri- and mature miRNA, hence showing an increase in pri-miRNA level and inhibiting the PDGF-BB-induced mature miR-193a (**Figure 68B**).



**Figure 68: PDGF-BB and E2 differentially affect pri-miRNA and mature miRNA expression in HCASMCs.** Evidence that E2 attenuates the effects of PDGF-BB on pri- and mature miRNA expression. Cells were grown to 60% confluency in complete media prior to treatment with PDGF-BB and E2 (100 nM) for 3, 6 and 24 hr. Expression of pri-miRNA and mature miRNA of miR-221 (**Panel A**) and miR-193a (**Panel B**). Total RNA was extracted and relative pri-miRNA and mature miRNA levels were determined by RT-qPCR using TaqMan assays for pri-miR-221 and pri-miR-193a or the respective TaqMan miRNA assay. The results from pri-miRNAs were normalized to GAPDH and hPRT1 mRNAs. Mature miRNA expression was normalized to U48 and U49. n=3, \*p<0.05 compared to control.

### 5.8.3 Discussion

The aim of the experiments described in this chapter was to assess whether E2-ER $\alpha$  down-regulates miR-193a and miR-221 by inhibiting them at the transcriptional level. Therefore, we examined the expression of the pri-miRNAs after treatment with E2. In contrast to our expectations, E2 did not suppress the expression of the pri-miRNAs, neither in HUVECs nor in HCASMCs.

In HUVECs, the level of pri-miRNA after 24 hr treatment with E2 was slightly decreased, but there was no significant down-regulation. To exclude time dependent regulation of pri-miRNAs, their expression was also analyzed at an earlier time point, however, we obtained negative results. Since we observed decreased mature miRNA levels after 24 hr without significant alteration in pri-miRNA expression, we postulate that E2 inhibits procession of these miRNAs rather than their transcription.

Surprisingly, we found that PDGF-BB inhibited the expression of the pri-miRNAs in HCASMCs at every tested time point, although mature miRNA levels are induced by PDGF-BB. Our results are also contrasting with the findings from Davis et al., which demonstrated that both pri-miR-221 and mature miR-221 are up-regulated by PDGF-BB within 0.5 to 24 hr and that PDGF-BB induces this miRNA by increasing its transcription [223]. Interestingly, E2 reversed the effects of PDGF-BB on pri-miRNA, showing increased expression compared to PDGF-BB, recalling its inhibitory action on PDGF-BB-induced mature miRNA levels. Since E2 attenuated both the inhibitory effects of PDGF-BB on pri-miRNA expression and stimulatory effects of PDGF-BB on mature miRNAs, we postulate that PDGF-BB stimulates the maturation process of these miRNAs, depleting the pri-miRNA levels in sustenance for higher mature miRNA expression and that E2 restrains it. Some studies demonstrate that E2 inhibits miR-221 in MCF-7 breast cancer cells expression via negative regulation of its transcription [291, 327, 328]. Moreover, E2-ER $\alpha$  induces miR-203 transcription in vascular SMCs via ERE-independent mechanisms, providing evidence that ERE in the promotor region of the miRNA gene are not a prerequisite for E2 dependent regulation of transcription [94]. However, we could not demonstrate that E2 inhibits miR-193a and miR-221 transcription in HUVECs and HCASMCs and therefore we suggest that it influences miRNA procession. Nevertheless, further research is necessary to test our assumption and clarify the mechanisms by which E2 inhibits miR-193a and miR-221.

In support of our hypothesis, ER $\alpha$  is able to regulate the biogenesis of miRNAs. Several components of the Drosha-processing complex interact with transcription factors, including SMADs and ER $\alpha$  [86, 193]. It was shown that E2-ER $\alpha$  directly inhibits pri-miRNA processing by Drosha, through interaction with p68 and p72, which are part of the Drosha microprocessor complex [330] and established ER $\alpha$  coregulators [331]. Indeed, Drosha and p68/DDX5 could be co-purified with ER $\alpha$  in MCF-7 cells [85]. E2-ER $\alpha$ -p68 interaction was reported to inhibit the formation of Drosha complex and thus repress pri-miRNA processing [332], though this work has been retracted [333]. Exportin-5, which controls the nuclear export of precursors, is induced by E2 [334] and processing of pre-miRNA to mature miRNA might also be coupled with ER $\alpha$ . The RNA co-activator steroid receptor RNA activator (SRA) binds Dicer complex components in various cell lines and also binds nuclear receptors, including ER $\alpha$ . Moreover, Dicer acts as nuclear receptor coactivator in MCF-7 cells [335] and its expression was shown to be induced by E2 and is higher in ER $\alpha$  positive breast cancers compared to negative ones [336, 337]. Finally, there is contrasting evidence that the expression of AGO2, the catalytic component of the RISC complex, is regulated by E2 both negatively, through activation of EGFR/MAPK signaling [338], and positively [336].

In summary, here we provide evidence that E2 does not inhibit miR-193a and miR-221 transcription in HUVECs and HCASMCs. Moreover, based on the recent findings that E2-ER $\alpha$  plays a role for Drosha and Dicer function and that E2 regulates AGO2, we postulate that E2 down-regulates miR-193a and miR-221 by inhibiting their procession from primary transcript to mature functional miRNAs.

## 5.9 Differential effect of miR-193a and miR-221 in vascular ECs and SMCs

### Objectives

E2 exerts differential effects on ECs and vascular SMCs; however, the mechanisms involved are not fully understood. Here we postulate that the differential actions of E2 are, in part, mediated via the down-regulation of miR-193a and miR-221. Hence, in this section we provide a summary of the cell specific effects of these miRNAs in HUVECs and HCASMCs and of their role in mediating the vascular actions of E2.

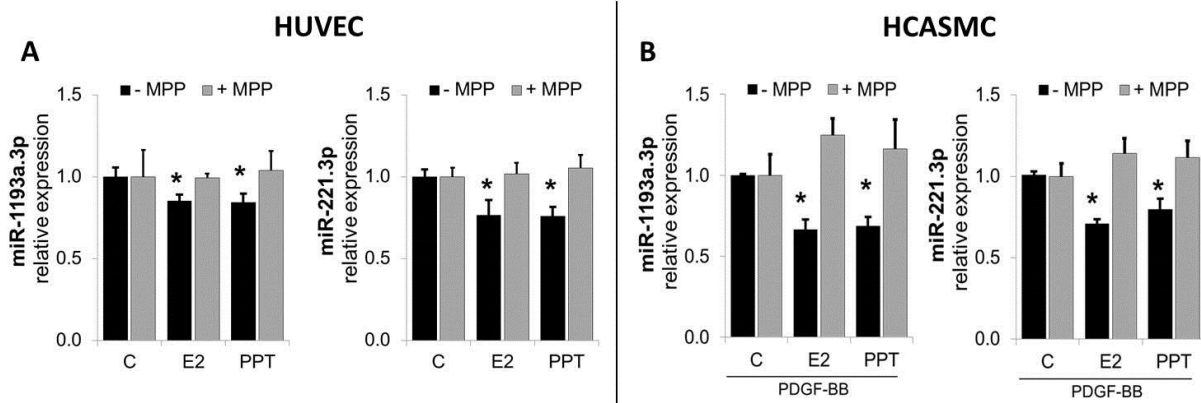
### Introduction

Impaired endothelial function and abnormal growth of vascular SMCs contribute to the vascular remodeling processes associated with CVD. The beneficial effects of estrogens are well established and include accelerated endothelial repair and regeneration and attenuation of intimal thickening. These effects are largely due to the differential effects of E2 in vascular cells, where it stimulates EC activity and inhibits vascular SMC growth [129]. However, the mechanisms involved in the differential role of E2 in vascular cells remain largely unknown. Recent studies in cancer cells provide evidence for cell specific effects of miRNAs [224, 225]. These cell specific actions of miRNAs appear to be relevant in the vascular system as well, where miRNAs have been demonstrated to differentially regulate EC and SMC growth and function [220, 226-230].

Since miR-193a and miR-221 are down-regulated by E2 in both HUVECs and HCASMCs and exhibit cell specific effects in vascular cells, we hypothesize that they mediate the differential actions of E2. Hence, in this section we summarize the cell specific differential effects of miR-193a and miR-221 and their role in mediating the protective actions of E2 on the vasculature.

#### 5.9.1 E2 down-regulates miR-193a and miR-221 via ER $\alpha$

In this study we found that E2 significantly down-regulates miR-193a and miR-221 in both HUVECs and PDGF-BB-induced HCASMCs. Moreover, we demonstrate that the inhibitory effects of E2 requires ER $\alpha$ , since the ER $\alpha$  agonist PPT reduced miRNA levels similar to E2 and the ER $\alpha$  antagonist PPT abrogated their effects ( $p < 0.05$ ; **Figure 69A and 69B**).

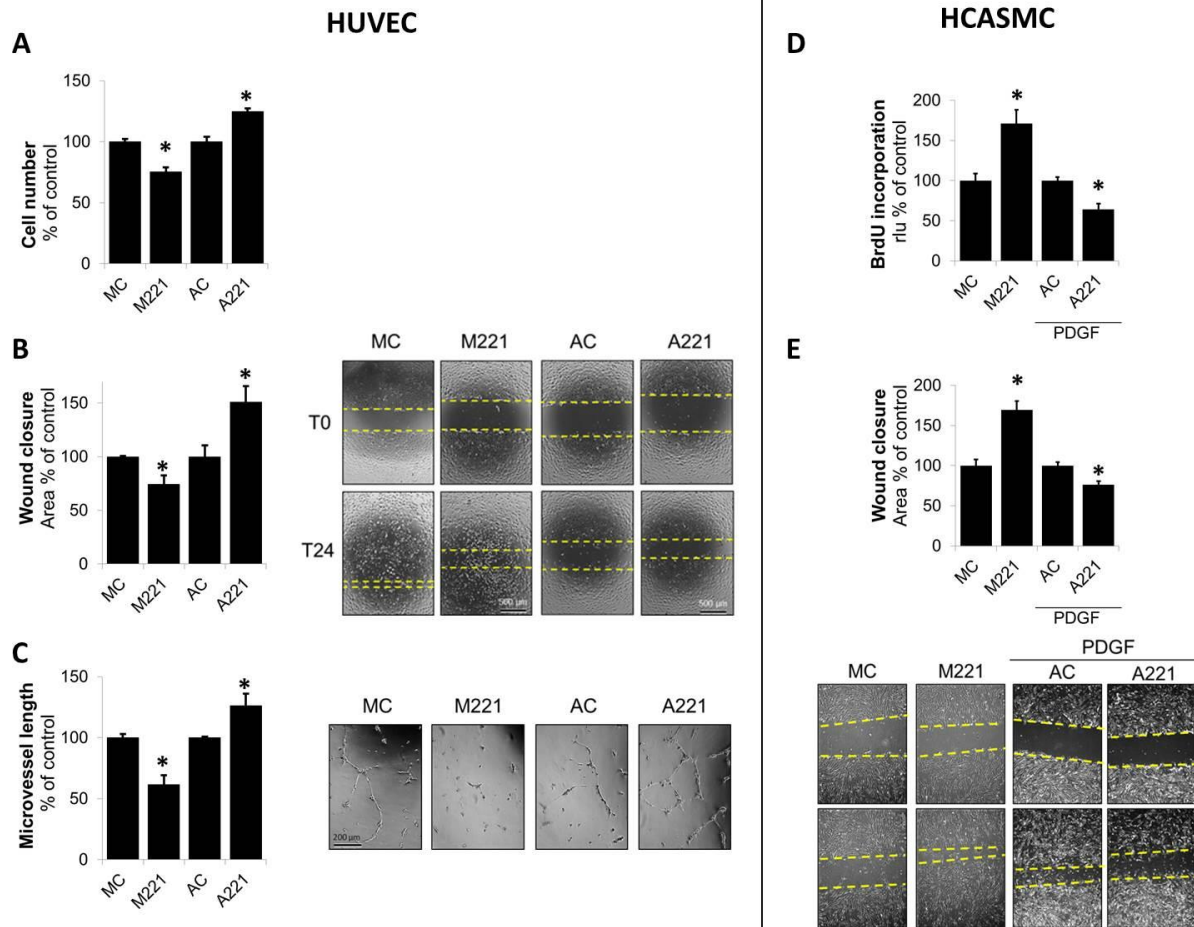


**Figure 69: E2 down-regulates miR-221 and miR-193 via ER $\alpha$  in HCASMCs and HUVECs.** Cells were incubated 24 hr with E2 or the ER $\alpha$  agonist PPT (100 nM) with or without the ER $\alpha$  antagonist MPP (500 nM). **Panel A:** HUVECs were treated with 10 nM E2. **Panel B:** HCASMCs were treated with 100 nM E2 in the presence of PDGF-BB (20 ng/ml). RNA was extracted and relative miRNA expression levels were determined by RT-qPCR using TaqMan miRNA assays. The results were normalized to U48 and U49. n=3, \*p<0.05 compared to control.

### 5.9.2 miR-193a and miR-221 differentially regulate ECs and SMCs

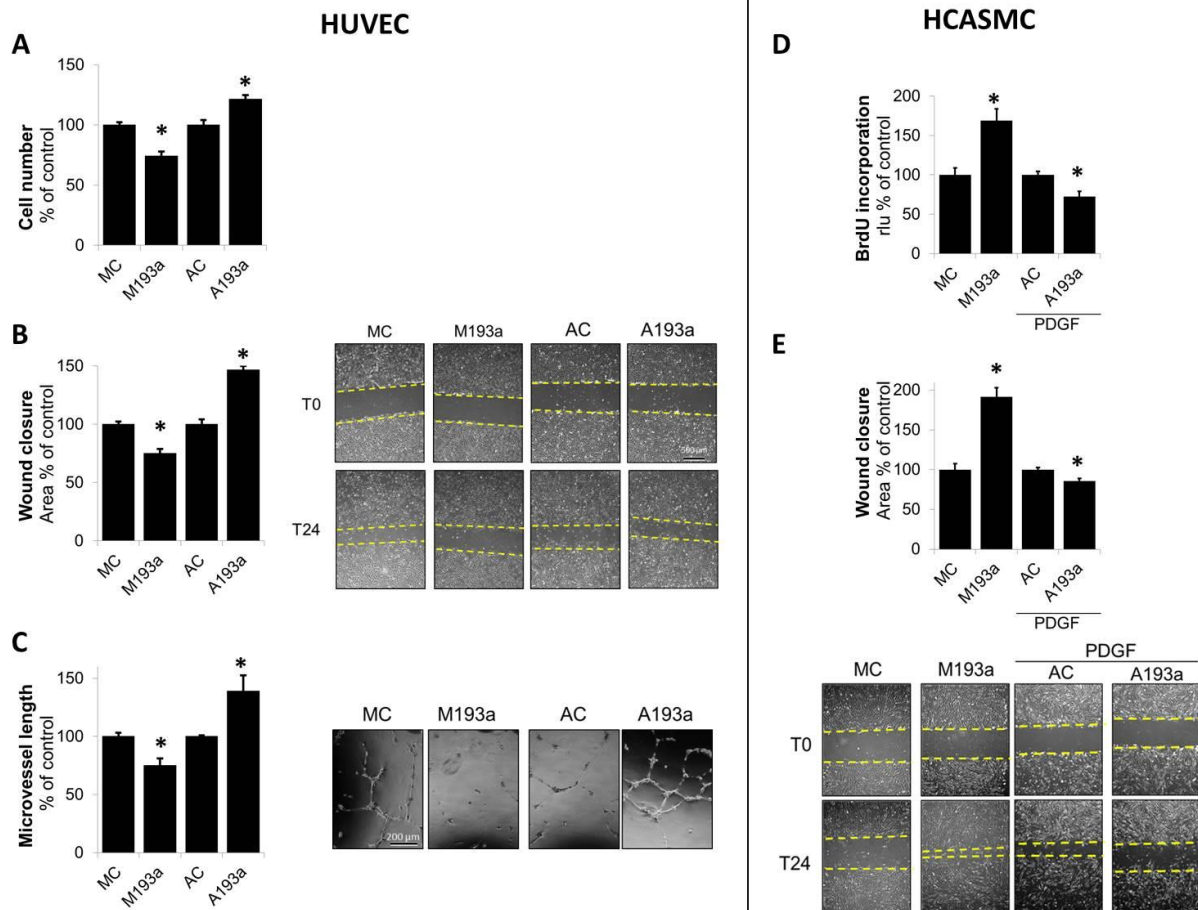
Since E2 has differential effects on EC and vascular SMC function and inhibits miR-193a and miR-221 in both HUVECs and HCASMCs, we hypothesized that these miRNAs might differentially regulate vascular cells. Indeed, using gain- and loss- of function approaches we found that miR-221 significantly inhibits proliferation, wound closure and tube formation in HUVECs (**Figure 70A, 70B and 70C**), while it promotes DNA synthesis and migration of HCASMCs (**Figure 70D and 70E**).





**Figure 70: Cell specific effects of miR-221 in HUVECs and HCASMCs.** HUVECs and HCASMCs were transfected with miR-221 Mimic (M221) or Antimir (A221) or their respective Controls (MC or AC) before tested in the different functional assays. HUVEC proliferation was assessed by cell counting (**Panel A**), migration by wound closure assay (**Panel B**) and tube formation using a matrigel-based assay (**Panel C**). HCASMCs proliferation was assessed by BrdU incorporation assay (**Panel D**) and migration by wound closure assay (**Panel E**).  $n=3$ ,  $*p<0.05$  compared to the respective control.

Similar to miR-221, miR-193a overexpression significantly inhibited proliferation, wound closure and tube formation in HUVECs, while it promotes DNA synthesis and migration of HCASMCs. In contrast, inhibition of miR-193a by the Antimir induced HUVEC proliferation, migration and vasculogenesis, while it inhibited HCASMC proliferation and migration (**Figure 71A-E**).

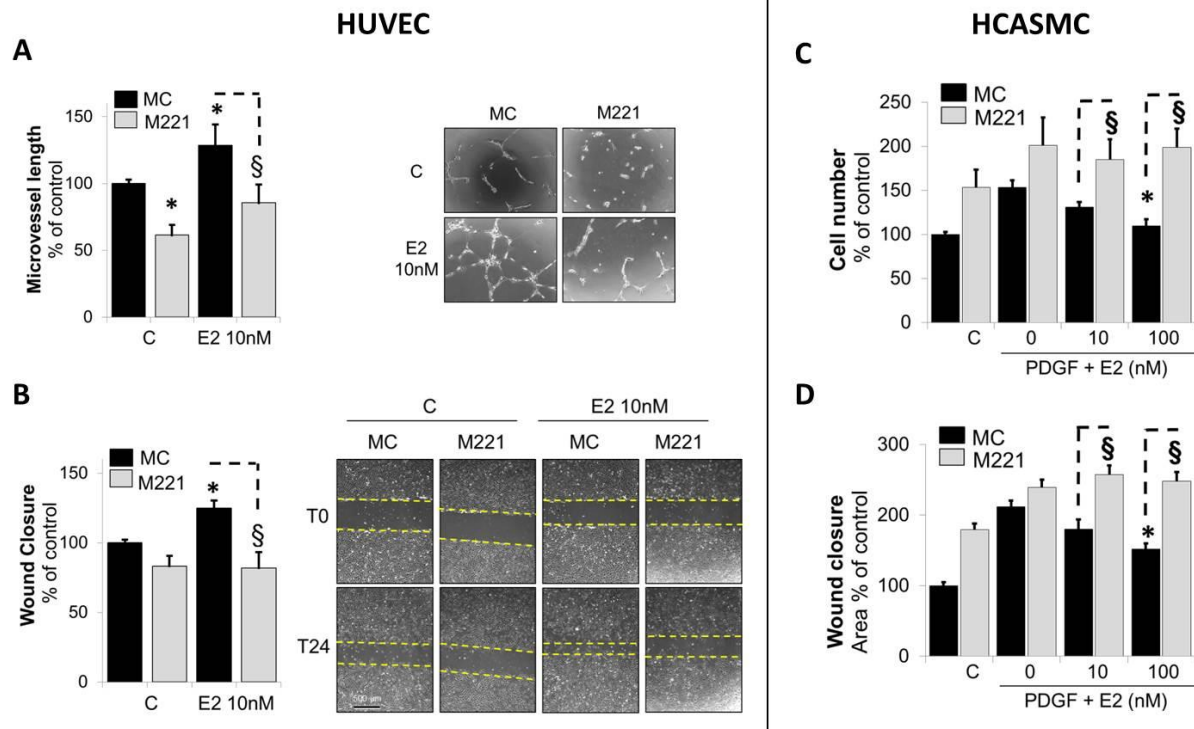


**Figure 71: Cell specific effects of miR-193a in HUVECs and HCASMCs.** HUVECs and HCASMCs were transfected with miR-193a Mimic (M193a) or Antimir (A193a) or their respective Controls (MC or AC) before being tested in the different functional assays. HUVEC proliferation was assessed by cell counting (**Panel A**), migration by wound closure assay (**Panel B**) and tube formation using a matrigel-based assay (**Panel C**). HCASMCs proliferation was assessed by BrdU incorporation assay (**Panel D**) and migration by wound closure assay (**Panel E**).  $n=3$ ,  $*p<0.05$  compared to the respective control.

### 5.9.3 Down-regulation of miR-193a and miR-221 by E2 mediates its differential actions on vascular cells

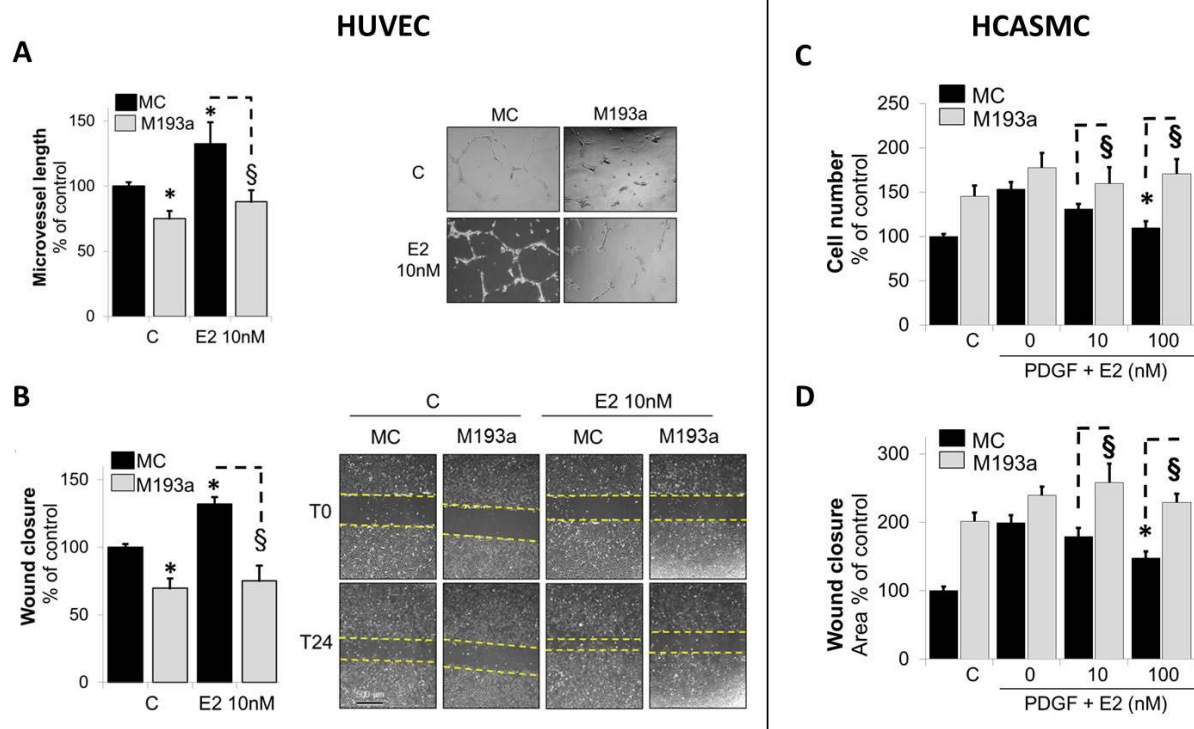
Since we demonstrated that E2 inhibits miR-193a and miR-221 and that these miRNAs have contrasting effects in HUVECs and HCASMCs, we hypothesized that the differential effects of E2 are mediated via the down-regulation of miR-193a and miR-221. Therefore, we investigated whether restoring the miRNA level after treatment with E2 reverses its stimulatory actions in HUVECs and inhibitory effects in HCASMCs. Indeed, we found that miR-221 overexpression abrogates E2-induced vasculogenesis and wound closure in HUVECs ( $p<0.5$ ; **Figure 72A and 72B**).

Moreover, up-regulation of miR-221 significantly reversed the inhibitory action of E2 on HCASMCs proliferation and migration (**Figure 72C and 72D**).



**Figure 72: miR-221 mediates the differential effects of E2 in HUVECs and HCASMCs.** HUVECs and HCASMCs were transfected with miR-221 Mimic (M221) or the respective Control (MC) in the presence or absence of E2 before being tested in the different functional assays. HUVEC tube formation was assessed using a matrigel-based assay (**Panel A**) and migration by wound closure assay (**Panel B**). HCASMC proliferation was assessed by BrdU incorporation assay (**Panel C**) and migration by wound closure assay (**Panel D**).  $n=3$ , \* $p<0.05$  compared to the respective control, § $p<0.05$  as indicated.

We observed similar results when restoring miR-193a levels after E2 treatment. Up-regulation of miR-193a significantly abrogated E2-induced tube formation and wound closure in HUVECs ( $p<0.05$ ; **Figure 73A and 73B**). Moreover, overexpression of miR-193a in HCASMCs significantly reversed the PDGF-BB-induced anti-mitogenic and anti-migratory actions of E2 (**Figure 73C and 73D**).



**Figure 73: miR-193a mediates the differential effects of E2 in HUVECs and HCASMCs.** HUVECs and HCASMCs were transfected with miR-193a Mimic (M193a) or the respective Control (MC) in the presence or absence of E2 before tested in the different functional assays. HUVEC tube formation was assessed using a matrigel-based assay (**Panel A**) and migration by wound closure assay (**Panel B**). HCASMC proliferation was assessed by BrdU incorporation assay (**Panel C**) and migration by wound closure assay (**Panel D**).  $n=3$ , \* $p<0.05$  compared to the respective control, § $p<0.05$  as indicated.

### 5.9.4 Discussion

In this chapter we summarize the cell specific actions of miR-193a and miR-221 in HUVECs and HCASMCs and their role in mediating the effects of E2. First, we showed that both miRNAs are inhibited by E2 in HUVECs and HCASMCs and we demonstrate that their down-regulation requires ER $\alpha$ . Second, we confirmed the dual role of miR-221 in vascular cells, where its up-regulation inhibits HUVEC proliferation, migration and tube formation and induces HCASMCs growth and migration. Moreover, suppression of miR-221 has opposite effects in both cell types. Third, we provide the first evidence that miR-193a plays an active role in the regulation of EC and vascular SMC functions and exhibits cell specific effects, similar to miR-221. Given the fact that E2 regulates both miRNAs and that their reduction mediates the actions of E2 in both HUVECs and HCASMCs, we postulate that the differential actions of E2 are due to the opposite effect of miR-193a and miR-221 in vascular

cells. Indeed, restoration of miRNA levels by overexpressing them abrogated E2-induced vasculogenesis and wound closure in HUVECs and reversed the inhibitory actions of E2 on HCASMC proliferation and migration. This supports our contention that both miR-193a and miR-221 participate in E2-mediated stimulation of ECs and inhibition of vascular SMCs.

Estrogens inhibit proliferation of vascular SMCs [124-127, 178, 180, 191] and have growth stimulatory and anti-apoptotic effects in ECs [130-136, 295]. Moreover, they enhance the proliferation of breast and uterine cells [296], indicating that E2 modulates diverse cell functions in a tissue specific manner. The specific actions of E2 are mediated via the ERs. Besides the differences in structure and ligand-binding specificity, the distribution of the ERs is also dissimilar depending on the tissue and more specifically the cell type [4]. Moreover, knock-out studies for ER $\alpha$  and ER $\beta$  provide evidence of different functions for the two receptors [22], hence indicating that the final effect of ER-ligands depends on ER regulation within a cell type, which is influenced by extracellular signals. Furthermore, E2 itself has been shown to regulate the expression of the ERs in many cell types, such as human breast cancer cells [23], retinal pigment epithelium [24], osteoclasts [25] and vascular SMCs [14].

Recently, estrogen regulated miRNAs have been shown to target ER $\alpha$  mRNA and influence its expression [316], suggesting that they may play a role in mediating the differential actions of E2. The cell specific effects of miRNAs have been shown in many cells [224, 225], including the vascular system, where miRNA such as miR-21, miR-26a and miR-221 differentially regulate EC and SMC functions [220, 226-230]. In this study we show that miR-221 and miR-193a have inhibitory actions on HUVEC proliferation, migration and tube formation while they induce HCASMC growth and migration. Moreover, we demonstrate that E2 interaction with ER $\alpha$  down-regulates miR-193a and miR-221 and provide evidence that miR-193a and miR-221 down-regulation by E2-ER $\alpha$  mediates the cell specific actions of E2 in ECs and vascular SMCs.

The molecular mechanisms by which these miRNAs exert their dual role is currently unclear and beyond the scope of this study. However, abundance of target mRNAs may be dependent on the cell type, as it is well established that gene expression profiles vary among different cells and environmental context. Therefore, it is feasible that the same miRNA has a great impact on its target in cells where the target is

highly expressed, compared to cells where this target is less abundant. Moreover, a single miRNA can regulate a whole set of different mRNAs and different miRNAs can target the same mRNA [67]. Thus, it is quite possible that target mRNAs compete for the binding of one miRNA and that many miRNAs compete for the binding of one specific target, given that binding occurs at the same region. Hence, the final effect of one miRNA may be strongly dependent on the relative abundance of its targets and on the expression levels of other miRNAs competing for the binding of the same target.

## 6 Conclusion and Perspectives

The observation that premenopausal women have lower incidence of CVD in comparison to age-matched men and postmenopausal women, suggest that estrogens protect against cardiovascular disorders [108-111]. This association is further supported by the fact that postmenopausal women taking hormone replacement therapy present a reduced risk for CVD and all-cause mortality [112-115]. Estrogens prevent vascular remodeling processes associated with CVD by promoting endothelial recovery and regeneration through induction of EC growth, recruitment of EPC and stimulation of angiogenesis [131, 132, 135, 136]. Moreover, estrogens inhibit vascular SMC proliferation and migration, thus preventing intimal thickening and vascular occlusion [125-127]. However, the underlying mechanisms for the vaso-protective actions of estrogens have not been completely elucidated. Therefore, the overall goal of this study was to improve our understanding of the mechanism(s) by which E2 regulates growth and function of vascular cells. These insights may be important for the development of drugs able to exhibit the desired beneficial effects of estrogens without inducing its undesired side effects, such as feminization and carcinogenicity. Since miRNAs are entailed in the processes of vascular remodeling [193-195] and estrogen signaling [316], we hypothesized that E2-regulated miRNAs participate in mediating the protective actions of E2 on the vascular system. Moreover, they could potentially be responsible for its differential effects on EC and vascular SMC growth.

We demonstrate that E2 down-regulates miR-193a and miR-221 in HUVECs. Moreover, suppression of miR-193a and miR-221 improves endothelial function, as it stimulates vasculogenesis, migration and proliferation of HUVECs. Further, our findings provide evidence that down-regulation of these miRNAs by E2 mediates the protective effects of E2. In addition, we show that miR-221 and miR-193a negatively influence EC functions by suppressing PI3K/ Akt / VEGF signaling and inhibiting the ALK1/ SMAD1/5/8/ Id1 pathway, which are both partly responsible for E2-stimulated tube formation in HUVECs.

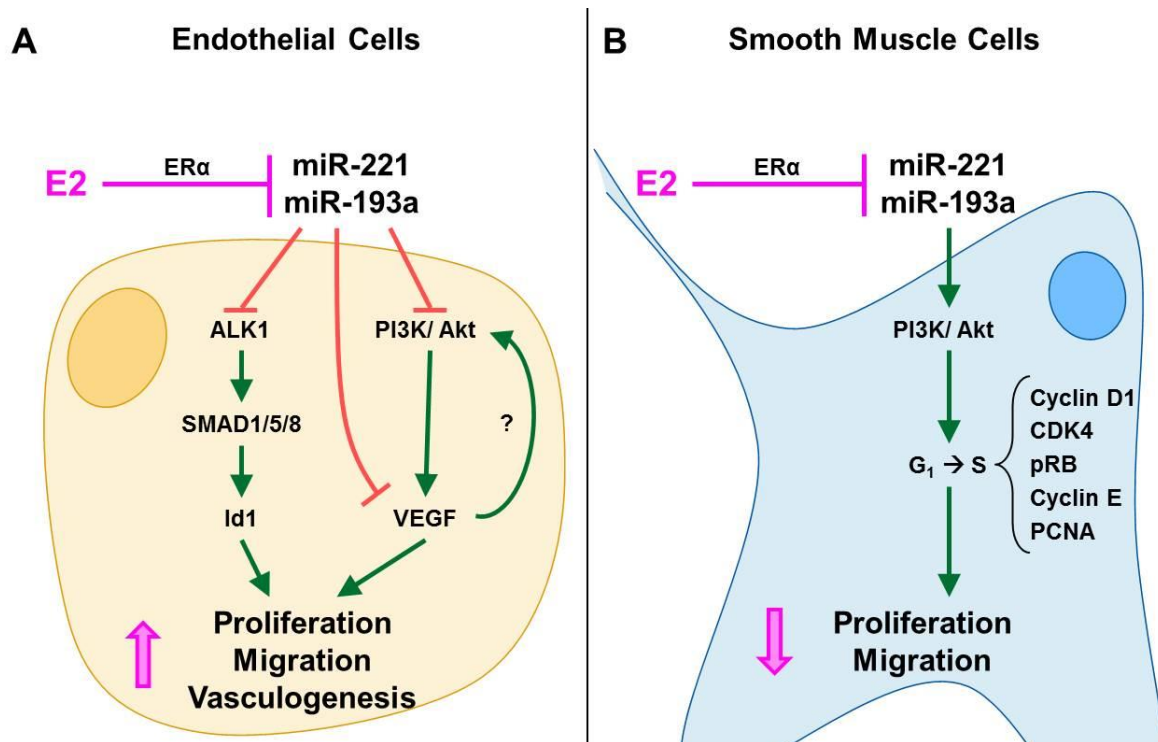
We determined that E2 down-regulates PDGF-BB-induced miR-193a and miR-221 in HCASMCs and that these miRNAs stimulate HCASMC proliferation and migration similar to PDGF-BB, while their suppression inhibits PDGF-BB-induced growth.



Moreover, we show that down-regulation of both miRNAs by E2 mediates the protective effects of E2 by inhibiting HCASMCs growth and migration. Additionally, we provide evidence that miR-193a and miR-221 induce cell cycle progression from G<sub>1</sub> to S phase, and inhibition of their expression by E2 or their respective Antimirs contributes to their anti-mitogenic actions in vascular SMCs.

Importantly, we provide the first evidence that miR-193a plays a role in differentially regulating EC and vascular SMC activity.

Taken together, we demonstrate that miR-193a and miR-221 actively contribute to the protective actions of E2 in the vascular system. Importantly, down-regulation of miR-193a and miR-221 by E2 improves endothelial function and inhibits vascular SMC proliferation and migration (**Figure 74**).



**Figure 74: Schematic representation of the role of miR-193a and miR-221 in mediating the protective actions of E2 and the potentially involved molecular mechanisms. Panel A:** Down-regulation of miR-221 and miR-193a suppresses their inhibitory effect on HUVEC functions. **Panel B:** Down-regulation of miR-221 and miR-193a by E2 suppresses their stimulatory effects on HCASMC proliferation. *E2*, 17β-estradiol; *ER*, Estrogen Receptor; *ALK1*, Activin Receptor-Like Kinase 1; *Id1*, Inhibitor of Differentiation; *PI3K*, Phosphoinositide 3 Kinase; *Akt*, Protein Kinase B; *VEGF*, Vascular Endothelial Growth Factor; *G<sub>1</sub>*, Gap1; *S*, Synthesis; *CDK*, Cyclin-Dependent Kinase; *pRB*, hyperphosphorylated Retinoblastoma protein; *PCNA*, Proliferating Cell Nuclear Antigene.



Furthermore, our findings suggest that miR-221 and miR-193a may be interesting therapeutic targets to protect post-menopausal women against occlusive vascular disorders. Since miR-221 and miR-193a are expressed in both genders, they might also be useful to treat CVD in men. However, it is yet unknown whether modulation of these miRNAs bypasses the cancerogenic side effects of hormones currently used for replacement therapy. The expression of miR-221 is different in ER positive and negative breast cancers and high miR-221 levels associate with tamoxifen and fulvestrant-resistance. Moreover, there is evidence that miR-221 contributes to breast cancer tumorigenesis through promotion of proliferation and metastasis [92, 291, 327]. In regard to miR-193a, numerous studies revealed that it suppresses tumor development and migration in different types of cancers, including breast and ovarian cancers [238-247], suggesting that further research is required to fully understand the role of both these miRNAs in the human body.

Compared to the classic molecular therapeutics, miRNA therapeutics is a promising field, with the advantage of small size and low molecular weight, conserved sequences and stability in the body fluids. Moreover, as miRNAs have multiple related targets, entire pathways involved in the pathogenesis of a disease may be modulated by the alteration of single miRNAs. The function of miRNAs can be restored or inhibited through the delivery of synthetic miRNA Mimics or Antimirs, respectively, or using miRNAs encoded in expression vectors. However, several obstacles including miRNA stability, inefficient and unspecific delivery, off-target effects and the immunogenicity of both the nucleic acids and delivery vehicles still have to be resolved.

The first strategy developed for systemic delivery of miRNAs *in vivo* is to use chemically modified Mimics and Antimirs to improve efficient and safe systemic delivery. 2'OH group modifications, such as 2'-O-methyl (2'-OMe), 2'-O-methoxyethyl (2'-MOE) and 2'-fluoro (2'-F), and locked nucleic acid (LNA) enhance stability, increase binding affinity and improve gene silencing compared to unmodified oligonucleotides. Moreover, increased nuclease resistance is achieved using peptide nucleic acid (PNA) and by substituting phosphodiester (PO) backbone linkage with phosphorothioate (PS) linkage, which additionally enhances the pharmacokinetic properties, facilitating delivery into many peripheral tissues. Modifications of the passenger strand of the double stranded miRNA Mimics prevents RISC loading and

protects against non-specific immune responses, while cellular uptake of the Mimics and Antimirs is facilitated by cholesterol conjugation. The second strategy is to restore miRNAs levels through viral delivery of expression vectors carried by viruses such as lentiviruses, adenoviruses and adeno-associated viruses. The application of tissue-specific promoters enables tissue-specific expression of miRNA Mimics or Antimirs and therefore to reduce off-target effects. The third strategy consists of delivering the miRNAs nanoparticles, designed to increase passive diffusion into target tissues. Liposomes and polymer-based nanoparticles protect miRNA Mimics and Antimirs from degradation and enhance their cellular uptake. Inorganic nanoparticles made of materials such as gold, carbon and silica, offer potential for the development of a biocompatible, non-immunogenic and non-toxic delivery system. Additionally, surface modifications of the nanoparticles using tissue- or tumor- specific antibodies allow specific delivery and may facilitate the internalization of the nanoparticle through receptor-mediated endocytosis.

The successful use of miRNA therapeutics has been demonstrated against several cardiovascular and metabolic disorders and tumors. Indeed, a small number of miRNA inhibition and replacement strategies to restore the original miRNA expression levels have already entered preclinical and clinical trials and may be soon available for clinical use, suggesting that the modulation of miRNAs may be a promising technology for future therapeutic developments [78, 339-343] .

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